Quantification of natural attenuation using analytical-chemical tools

Reitzel, Lotte

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Lotte Ask Reitzel

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Preface

This thesis comprises the research done for a Ph.D. project undertaken from 2000 to 2005 at Environment & Resources DTU, Technical University of Denmark. The primary supervisor was Professor Poul L. Bjerg and the co-supervisor was Associate professor Anna Ledin, Environment & Resources DTU. The project was funded by the Technical University of Denmark.

The thesis is composed of a summary of the subject "Quantification of natural attenuation using analytical-chemical tools" along with 7 journal papers, 4 published and three submitted for publication, with the in-text reference numbers **I-VII**:

I Reitzel, L.A. and Ledin, A. (2002) "Determination of phenols in landfill leachate-contaminated groundwaters by solid-phase extraction", Journal of Chromatography A 972, 175-182

II Baun, A., Reitzel, L.A., Ledin, A., Christensen, T.H., Bjerg, P.L. (2003) "Natural attenuation of xenobiotic organic compounds in a landfill leachate plume (Vejen, Denmark)", Journal of Contaminant Hydrology 65, 269-291

III Richnow, H.H., Meckenstock, R.U., Reitzel, L.A., Baun, A., Ledin, A., Christensen, T.H. (2003) "In situ biodegradation determined by isotope fractionation of aromatic hydrocarbons in an anaerobic landfill leachate plume (Vejen, Denmark)", Journal of Contaminant Hydrology 64, 59-72

IV Reitzel, L.A., Tuxen, N., Ledin, A., Bjerg, P.L. (2004) "Can degradation products be used as documentation for natural attenuation of phenoxy acids", Environmental Science & Technology 38, 457-467

V Ledin, A., Reitzel, L.A., Bjerg, P.L. (2005) "Quantitative determination of toluene, ethylbenzene and xylene degradation products in contaminated groundwater by solid phase extraction and in-vial derivatization", manuscript

VI Reitzel, L.A., Juhler, R.K., Tuxen, N., Ledin, A., Bjerg, P.L. (2005) "Quantification of in situ biodegradation based on changes in enantiomeric fraction of chiral compounds", manuscript

VII Reitzel, L.A., Bjerg, P.L., Juhler, R.K. (2005) "Enantiomer-specific analysis of MCPP, dichlorprop, and their phenoxy acid impurities in groundwater using liquid chromatography – tandem mass spectrometry (LC-MS/MS)", manuscript

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Lotte Ask Reitzel

N.B.: The papers are not included in this www-version, but can be obtained from the Library at Environment & Resources DTU, Bygningstorvet 115, Technical University of Denmark, DK-2800 Lyngby (library@er.dtu.dk).

Abstract

Contamination of groundwater is a widespread environmental problem, which particularly arises from various point sources such as leaking underground storage tanks, accidental spills, inappropriate use and disposal techniques, and industrial discharges. Thus, the development of efficient and cost-effective remediation approaches is urgently needed. The natural attenuation concept is a passive approach relying on the ability of the intrinsic microorganisms to degrade the contaminants. This approach implies the demonstration and verification of on-going biodegradation and a subsequent monitoring. However, the verification of biodegradation is not straightforward, since decreasing concentrations are ambiguous, and direct field scale mass reduction can be difficult to prove. Various *in situ* indicators have been suggested as supplemental evidence in the demonstration of biodegradation. A number of these *in situ* indicators are evaluated herein.

The evaluation emphasized the importance of differentiating between bulk contamination and specific compounds. The term, bulk contamination, is used to describe a mixture of contaminants, which make up the majority of the plume and can be metabolically degraded. The degradation of bulk contamination may significantly alter the groundwater quality in terms of exhaustion of electron acceptors and production of DIC and alkalinity. This phenomenon is typically observed at hydrocarbon field sites and landfills.

A specific compound is basically any compound, which is considered separately, but the term is especially used to describe specific contaminants of special environmental concern. The degradation of a specific compound is not reflected in the bulk changes of e.g. concentrations of electron acceptors, either because the concentration of the specific compound (e.g. pesticides or critical components of petroleum such as benzene) is possibly orders of magnitude lower than the overall concentration of bulk contamination, or because the specific compound (e.g. chlorinated solvents and other highly chlorinated compounds) is degraded by reduction and therefore is related to the consumption of an electron donor rather than the consumption of electron acceptors.

The use of degradation products for the demonstration of biodegradation was investigated. The starting point was the traditional use of dechlorination products to verify the occurrence of reductive dechlorination of chlorinated solvents such as perchloroethene (PCE) and trichloroethene (TCE). The sequential and quantitative transformation of parent compounds into successively less chlorinated daughter compounds implies the possibility of quantification of biodegradation.

More recently, the use of benzyl- and alkylbenzylsuccinates were suggested as specific indicator metabolites of the anaerobic degradation of alkylbenzenes. It was found that these metabolites were likely to occur at hydrocarbon field sites and even

at landfill sites, but not in uncontaminated groundwater. However, the evaluation of existing field studies indicated that the succinate derivatives could only be used as qualitative indicators, while their possible use for quantification was questionable.

Degradation products are known for a number of pesticides. The phenoxy acid herbicides are frequent groundwater contaminants with a well-described degradation pathway going through the corresponding chlorophenol. A few degradation studies also showed dechlorination as a possibility under anaerobic conditions. The chlorophenols and the possible dechlorination products are also frequently found in groundwater, which at first might suggest *in situ* degradation of phenoxy acids. However, a review of the history of the manufacture of these herbicides revealed that a range of chlorophenols and non-herbicide phenoxy acids are present as impurities in the herbicides. In the early years of production the impurities could account for more than 30% of the herbicide. Therefore, unless a direct increase in a metabolite is observed along a flowline, the presence of possible phenoxy acid degradation products in contaminated groundwater does not indicate the degradation of phenoxy acids.

It was found, however, that the impurity/parent herbicide ratios could be used as *in situ* indicators. Indication of biodegradation is obtained when the impurity/parent compound ratio changes along a flowline, although in the case of chlorophenol impurities, the change needs to be an increase, since a decreasing ratio could be caused by sorption of the chlorophenol. Indication of biodegradation can also be obtained independently of the flowpath, if the impurity/parent herbicide ratio exceeds the worst-case ratio, which is the largest possible ratio in the original herbicide based on synthesis yields.

The use of specific compound isotope analyses has gained much interest in recent years. The degradation of an organic compound may result in an increasing $^{13}C/^{12}C$ ratio of the residual fraction due to isotopic fractionation. Enrichment factors have been determined for a number of compounds, primarily hydrocarbons and chlorinated hydrocarbons, under different redox conditions. This means that when degradation of a specific compound is indicated by an increasing isotope ratio of the compound along a flowline, the relative biodegradation can be quantified.

The carbon isotope fractionation seems to be somewhat larger for chlorinated solvents than for hydrocarbons, especially for the less chlorinated daughter compounds. But the concurrent formation and further degradation of these intermediate metabolites complicates the interpretation.

For hydrocarbons the approach is more easily applied, and has been successful even in a complex matrix of leachate-contaminated groundwater with relatively low concentrations of specific hydrocarbons. Yet, at most field sites the approach only indicated degradation for some of the specific compounds, while no systematic trend could be identified in the isotope ratios of other compounds. This could be because the degradation of the latter compounds at the specific field site was not associated

with isotope fractionation, or because the concentration dropped to below the detection limit of the isotope analysis.

Specific compound isotope analysis is not feasible for phenoxy acids, because the analysis involves the separation of compounds by GC, and derivatization should be avoided as it might cause fractionation and increases the analytical uncertainty by adding a substituent to the molecule, which needs to then be corrected. Some phenoxy acids, however, are chiral molecules, which might be enantioselectively degraded, depending on the environmental conditions. In this way the ratio between the enantiomers, i.e. the two mirror image forms, changes. Much like the isotope ratios, a change in enantiomeric composition along a flowline will indicate degradation.

An advantage of enantiomeric fractions compared to isotope ratios is a lower detection limit and that many sources can be assumed to have a racemic composition of the phenoxy acids. In that case, the observation in the plume of an enantiomeric fraction significantly different from racemic is indicative of degradation, independent of the flowpath.

It was found that a site-specific enantioselectivity could be estimated in supportive microcosm degradation studies, and subsequently applied to obtain quantitative estimates of biodegradation at the field scale based on the observed changes in enantiomeric composition. The enantioselectivities probably needs to be determined for each field site, while isotope enrichment factors for a certain compound under certain redox conditions seems to be more generally valid. However, the successful application of isotope ratios for phenoxy acids is currently not very likely.

Having quantified the degradation of some specific compounds based on the changes in isotope ratios or enantiomeric compositions, the degradation of some other compounds could be quantified as well by way of the use of compound ratios. A compound ratio is the ratio between two compounds with similar physical and chemical properties that can be related to the same part of the source, which is why the possible change in the compound ratio along a flowline indicates degradation. As stand-alone indicators, the compound ratios may have a relatively limited use as qualitative indicators, but in combination with other methods, they were found to be very useful tools.

Overall, the evaluated *in situ* indicators all have their forces and limitations. Some apply to the degradation of bulk contamination, while others apply to a single or a group of specific compounds. Some are qualitative, while others are quantitative. Some are flowpath dependent, while others are absolute. Each *in situ* indicator may provide a piece of the puzzle, and the combined evidence from different *in situ* indicators along with other lines of evidence may eventually form the basis for a safe and efficient application of natural attenuation as a remedy.

Resumé

Forurening af grundvandet er et udbredt miljøproblem, som ikke mindst skyldes en bred vifte af punktkilder, såsom utætte beholdere og rørledninger, spild, industrielle udledninger og uhensigtsmæssig håndtering og bortskaffelsesmetoder. Der er derfor et stort behov for udvikling af effektive og rentable afværgestrategier. "Naturlig nedbrydning" er en passiv metode baseret på de naturligt forekommende mikroorganismers evne til at nedbryde forureningen. Ved anvendelse af naturlig nedbrydning som afværgestrategi skal det verificeres, at der foregår nedbrydning af forureningen på lokaliteten, hvorefter et moniteringsprogram sættes i gang. Imidlertid er det ikke så enkelt at påvise nedbrydning i felten, eftersom faldende koncentrationer er tvetydige, og direkte massefjernelse er vanskelig at bevise. Som supplement er der foreslået en række forskellige *in situ* indikatorer. En del af disse evalueres her.

Evalueringen tydeliggjorde vigtigheden af at skelne mellem bulk forurening og specifikke stoffer. Benævnelsen "bulk forurening" bruges til at beskrive en blanding af stoffer, som udgør størstedelen af forureningsfanen i grundvandet, og som kan nedbrydes metabolisk. Nedbrydning af bulk forurening kan ændre signifikant på grundvandets sammensætning pga. forbrug af elektronacceptorer og production af uorganisk kulstof og alkalinitet. Dette fænomen optræder typisk i forbindelse med benzin- og olieforureninger samt lossepladser.

Et specifikt stof er basalt set ethvert stof, som betragtes separat, men benævnelsen bliver specielt brugt til at beskrive specifikke forureningskomponenter, som udgør en særlig trussel for miljøet. Nedbrydningen af et specifikt stof afspejles ikke i bulk parametre som f.eks. koncentrationen af elektronacceptorer, enten fordi det specifikke stof (f.eks. pesticider eller kritiske komponenter i benzin eller olie såsom benzen) typisk vil have en koncentration, der er størrelsesordener lavere end total koncentrationen af bulk forurening, eller fordi det specifikke stof (f.eks. chlorerede opløsningsmidler og andre høj-chlorerede stoffer) nedbrydes ved reduktion, og derfor ikke kan relateres til forbrug af elektronacceptorer, men snarere til forbrug af en elektrondonor.

Anvendelsen af nedbrydningsprodukter til påvisning af bionedbrydning blev undersøgt. Udgangspunktet var den traditionelle anvendelse af dechloreringsprodukter som bevis for, at der foregår reduktiv dechlorering af chlorerede opløsningsmidler såsom perchlorethylen (PCE) og trichlorethylen (TCE). Den sekventielle og kvantitative omdannelse af moderstoffer til stadigt mindre chlorerede nedbrydningsprodukter indebærer en mulighed for at kvantificere bionedbrydningen.

Inden for de senere år er benzyl- og alkylbenzylravsyrer blevet foreslået som specifikke indikator-metabolitter for anaerob nedbrydning af alkylbenzener. Feltundersøgelser samt et review af litteraturen indikerede, at disse metabolitter med god sandsynlighed kan måles i forureningsfaner fra benzin- eller olieforureninger og lossepladser, men ikke i uforurenet grundvand. Dog viste evalueringen, at

ravsyrederivaterne formentlig kun kan anvendes som kvalitative *in situ* indikatorer, men ikke til kvantificering af bionedbrydningen.

Der er identificeret nedbrydningsprodukter for en række pesticider. Phenoxysyre-herbiciderne er almindelige forureningskomponenter i grundvand med en velbeskrevet nedbrydningsvej, som går via den korresponderende chlorphenol. Enkelte nedbrydningsstudier har også vist at dechlorering kan være en mulighed under anaerobe forhold. Chlorphenolerne og de mulige dechloreringsprodukter er også almindelige forureningskomponenter i grundvand, hvilket umiddelbart kunne tyde på nedbrydning af phenoxysyrer. Men en gennemgang af produktionshistorien for disse herbicider afslørede, at herbiciderne indeholdt en række urenheder bestående af chlorphenoler samt phenoxysyrer uden selvstændig anvendelse. I herbicid-produktionens tidlige år kunne urenhederne tilsammen udgøre over 30% af herbiciderne. Dette betyder, at medmindre der ses en direkte stigning i metabolit-koncentrationen langs en flowlinie, vil tilstedeværelsen af mulige phenoxysyre-nedbrydningsprodukter i forurenet grundvand ikke indikere nedbrydning af phenoxysyrer.

Derimod viste det sig, at urenhed/moderstof-forholdene kunne anvendes som *in situ* indikatorer. Bionedbrydning kan påvises ved en ændring i urenhed/moderstof-forholdet langs en flowlinie. For chlorphenol-urenheder kan kun en stigning i urenhed/moderstof-forholdet utvetydigt relateres til nedbrydning, da et fald også kan forårsages af sorption af chlorphenolen. Alternativt kan bionedbrydning påvises ved at urenhed/moderstof-forholdet overstiger worst-case forholdet, som er det størst mulige urenhed/moderstof-forhold, som baseret på syntesen kan findes i det oprindelige herbicid.

Anvendelsen af isotopanalyser for specifikke stoffer er vundet frem i de senere år. Nedbrydningen af et organisk stof kan medføre en stigning i $^{13}C/^{12}C$-forholdet i den tilbageværende fraktion pga. isotopfraktionering. For et antal stoffer, primært kulbrinter og chlorerede kulbrinter, er enrichment-faktorer blevet bestemt under forskellige redox-forhold. En stigning i isotopforholdet for et stof langs en flowlinie vil således indikere, at stoffet bliver bionedbrudt, og ved hjælp af enrichment-faktoren kan bionedbrydningen kvantificeres.

Carbon isotopfraktioneringen lader til at være mere udpræget for chlorerede opløsningsmidler end for kulbrinter, specielt for de mindre chlorerede nedbrydningsprodukter. Men den samtidige dannelse og fjernelse af disse intermediære metabolitter gør fortolkningen vanskelig.

For kulbrinter er metoden forholdsvis enkel at anvende, og har givet fornuftige resultater selv i en kompliceret matrix som perkolat-forurenet grundvand med relativt lave kulbrinte-koncentrationer. På de fleste forurenede lokaliteter var det dog kun nedbrydningen af nogle af de testede stoffer, som kunne verificeres vha. isotopforhold, mens isotopforholdene for andre af stofferne ikke udviste en generel stigning med afstanden fra kilden. Dette kan skyldes, at nedbrydningen af disse stoffer

på lokaliteten foregik uden isotopfraktionering, eller at stoffernes koncentrationer faldt til under isotopanalysens detektionsgrænse.

For phenoxysyrer er det vanskeligt at lave isotopanalyser, da isotopanalysen kræver separering af stoffer vha. gaschromatografi, og derivatisering helst skal undgås pga. risikoen for isotopfraktionering under reaktionen, og fordi analyse-usikkerheden øges, da der skal korrigeres for den tilføjede substituent i molekylet. Imidlertid er nogle af phenoxysyrerne chirale molekyler, som under nogle forhold nedbrydes enantioselektivt, hvorved forholdet mellem de to enantiomerer (spejlbilled-former) ændres. Som det var tilfældet med isotopforhold, kan en ændring i enantiomer-forholdet langs en flowlinie indikere nedbrydning.

En fordel ved enantiomer-forholdene sammenlignet med isotop-forholdene er, at mange kilder kan antages at indeholde phenoxysyrerne i en racemisk blanding. I så fald vil et enantiomer-forhold i forureningsfanen, som er signifikant forskelligt fra det racemiske udgangspunkt, indikere nedbrydning uafhængigt af flow-vejen.

Det blev vist, at supplerende nedbrydningsstudier i laboratoriet kunne bruges til at bestemme en lokalitets-specifik enantioselektivitet, som dernæst kunne bruges til at kvantificere nedbrydningen baseret på ændringer i enantiomer-forholdet i felten. Det vil sandsynligvis være nødvendigt at bestemme enantioselektiviteten specifikt for hver feltlokalitet, mens enrichment-faktorer for isotopfraktioneringen for et bestemt stof under bestemte redox-forhold formentlig gælder mere generelt. Imidlertid er anvendelsen af isotop-forhold for phenoxysyrer ikke en reel mulighed i dag.

Har man først kvantificeret nedbrydningen af et eller flere specifikke stoffer udfra ændringen i isotop-forhold eller enantiomer-forhold, kan nedbrydning af lignende stoffer kvantificeres vha. ”stof-forhold”. Et stof-forhold er forholdet mellem to stoffer, som har omtrent samme fysiske og kemiske egenskaber, og som kan relateres til den samme kilde eller samme område i kilden. En ændring i stof-forholdet langs en flowlinie vil således indikere nedbrydning. Som enkeltstående indikatorer har stof-forholdene en begrænset anvendelighed som kvalitative indikatorer, men i kombination med andre metoder er de særdeles effektive.

Alt i alt har alle de evaluerede *in situ* indikatorer deres styrker og begrænsninger. Nogle retter sig mod nedbrydningen af bulk forurening, mens andre er rettet mod enkeltstående eller grupper af specifikke stoffer. Nogle er kvalitative, andre kvantitative. Nogle afhænger af flow-vejen, mens andre er absolutte. Hver *in situ* indikator kan potentielt bidrage til verificeringen af naturlig nedbrydning, og kombinationen af flere forskellige *in situ* indikatorer samt andre typer af værktøjer såsom modellering eller mikrobiologiske og molekylære teknikker kan give et samlet billede, der i sidste ende danner grundlaget for en vurdering af, om naturlig nedbrydning på en given lokalitet vil være en sikker og effektiv afværgestrategi.

Abbreviations

4-CPA	4-chlorophenoxyacetic acid
2-CPP	2-(2-chlorophenoxy)-propionic acid
4-CPP	2-(4-chlorophenoxy)-propionic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2,6-DCPP	2-(2,6-dichlorophenoxy)-propionic acid
2,6-MCPP	2-(6-chloro-2-methylphenoxy)-propionic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
BSA	Benzylsuccinic acid/benzylsuccinate
BTEX	Benzene, toluene, ethylbenzene and the xylene isomers
CAH	Chlorinated aliphatic hydrocarbons
Cis-1,2-DCE	*Cis*-1,2-dichloroethene
DCE	Dichloroethene
DIC	Dissolved inorganic carbon
Dichlorprop	2-(2,4-dichlorophenoxy)-propionic acid
Dichlorprop-P	*(R)*-2-(2,4-dichlorophenoxy)-propionic acid
DOC	Dissolved organic carbon
EA	Electron acceptor
ED	Electron donor
EF	Enantiomeric fraction
ER	Enantiomeric ratio
F	Dilution factor
GC	Gas chromatography
GC/C/IRMS	Gas chromatography/combustion/isotope ratio mass Spectrometry
HCH	1,2,3,4,5,6-hexachlorocyclohexane
HPLC	High performance liquid chromatography
IRMS	Isotope ratio mass spectrometry
K_{ow}	Octanol-water distribution coefficient
LC	Liquid chromatography
LNAPL	Light non-aqueous phase liquid
MCPA	4-chloro-2-methylphenoxyacetic acid
MCPP	2-(4-chloro-2-methylphenoxy)-propionic acid (mecoprop)
MCPP-P	*(R)*-2-(4-chloro-2-methylphenoxy)-propionic acid
MNA	Monitored natural attenuation
MTBE	Methyl-*tert*-butylether
RNA	Ribonucleic acid
PDB	Peedee Belemnite, carbonate standard for carbon isotope ratios
PCE	Perchloroethene

PCP	Pentachlorophenol
TCE	Trichloroethene
TMB	Trimethylbenzene
TPH	Total petroleum hydrocarbons
VC	Vinylchloride

1. Introduction

1.1 Background

Since the 1970s it has been recognized that pollution of groundwater is a widespread environmental problem [12], constituting a serious risk to the water supply, as groundwater is a crucial resource for drinking water in many countries. Various point sources contributing to this contamination can be identified such as leaking underground storage tanks and pipelines, accidental spills, inappropriate use and disposal techniques, and industrial discharges. This emphasizes a need for efficient remediation and protection strategies in order to ensure the availability of clean groundwater for future generations.

Traditional remediation systems, such as pump and treat, are expensive, and in a number of cases failed to reach the cleanup goals. Therefore, alternative approaches have gained more interest. Recently, remediation using monitored and enhanced natural attenuation has been recognized as cost-effective, risk-based, low-intensity technologies for the *in situ* treatment of contaminated land and groundwater. Monitored natural attenuation has been successfully applied to petroleum hydrocarbon spills, while the understanding of the natural attenuation of a wider range of contaminants is offered increasing interest, as well as the development of better tools for the demonstration and verification of natural attenuation [143].

1.2 Natural attenuation processes

Natural attenuation refers to all of the naturally occurring processes that contribute to the reduction of the toxicity, mobility, concentration or mass of a pollutant in soil and groundwater at a contaminated site [151]. These processes can be destructive or non-destructive, and include biodegradation, abiotic degradation, chemical stabilization, volatilization, sorption, dispersion and dilution. Natural attenuation in general occurs at any site, and might potentially prevent the pollutant from reaching down gradient receptors such as drinking water wells, surface waters or groundwater resources.

Biodegradation and abiotic degradation are the only truly destructive processes, which may ultimately lead to the complete breakdown of organic contaminants into harmless end-products. Depending on the environmental conditions, microorganisms may be able to completely mineralize an organic contaminant. Abiotic degradation on the other hand will in many cases only lead to a transformation of the contaminant, by for instance hydrolysis of acid derivatives or reduction of nitro-substituents to amino-substituents, while complete mineralization is less likely.

Volatilization is a non-destructive process, but for groundwater it is a mass-removing process, since contaminants are transferred to the atmosphere, where they

might be subject to e.g. photochemical degradation. Sorption and chemical stabilization (e.g. complexation, precipitation, ion exchange) can act as practically non-reversible processes, and are indeed the only valid processes for heavy metals [28], which are elements and therefore non-degradable. However, these processes only serve to contain the contaminants, and a change in the chemical conditions may reverse the processes. Thus, long-term efficiency is difficult to assure.

1.3 Monitored natural attenuation as a remedy

"Monitored natural attenuation" (MNA) is a remediation approach based on the reliance on the natural attenuation to remediate a contaminated site and protect down gradient receptors [151]. It can be used as a stand-alone technique as well as a follow-up remediation or complement of other techniques [142]. The term, MNA, suggests that any attenuation process is accepted, but the current practice is to rely more on the destructive and/or non-reversible processes to achieve a long-term recovery [151]. Other denominations such as "intrinsic bioremediation" or "natural assimilation" are also found in the literature. These denominations better reflect the fact that at the majority of sites contaminated with organic pollutants, biodegradation is the only process that can guarantee long-term efficiency and eventually the complete remediation of the site.

Monitored natural attenuation involves a detailed investigation and demonstration in order to prove the efficiency of the natural attenuation processes to reach the remediation goals. Furthermore, a long-term monitoring of the site is made, to ensure that the contamination is actually being reduced [151]. In the past 15 years, MNA has gained increasing acceptance in response to the progress of the understanding of the subsurface processes, the development and improvement of analytical approaches for the *in situ* demonstration of contaminant mass removal, as well as the development of better modeling tools. Since 1995, protocols or guidelines on MNA have been developed especially in the USA and more recently in the UK and the Netherlands [119,124,130,151,156,157].

1.4 Lines of evidence

For the demonstration of the natural attenuation processes, the protocols use the concept of "lines of evidence" [130]. This term refers to the different ways to prove or render probable that contaminant mass removal will remediate the site. The common approach is to use three lines of evidence. The exact differentiation between the three lines of evidence is not clear-cut and depends on the applied protocol.

First line of evidence

The first line of evidence is based on the analysis/interpretation of spatial and historical data series in order to identify a reduction of contaminant concentration

with distance from the source, and to evaluate if the contaminant plume is expanding, stable or shrinking. The identification of and differentiation between attenuation processes as well as the demonstration and/or quantification of mass removal can also be a part of the first line of evidence.

Second line of evidence

The second line of evidence consists of a demonstration of field-scale biodegradation based on *in situ* indicators. Several *in situ* indicators have been suggested, such as an observed depletion of electron acceptors and the generation of respiration by-products (e.g. Mn(II), Fe(II), CH_4 etc.) in correspondence to contaminant disappearance, or the occurrence of degradation products [157]. Recently, the identification of highly specific signature metabolites has gained attention [13,107]. Novel techniques such as the measurement of changes in isotope ratio [97,128] or enantiomeric fraction [106] may also add to the second line of evidence, but the experiences are relatively few. Contaminant mass balance calculations and contaminant flux calculations are made to eventually determine the extent of biodegradation or the degradation rate.

Third line of evidence

The third line of evidence includes microbiological evidence, which traditionally implies the demonstration of the degradation potential (e.g. in microcosm experiments or through the enumeration of specific degraders) as opposed to the verification of actual degradation at the field site. However, some recent molecular techniques provide new possibilities. These techniques include the detection of specific genes (coding for enzymes associated with particular degradation pathways) or their specific expressions (RNA or proteins), and the direct identification of specific microorganisms by their genetic features [36,135]. While the detection of specific genes or specific micro-organisms is an alternative way of demonstrating degradation potential, the detection of RNA or proteins serves as direct proof of on-going degradation.

Flow and reactive transport modeling of field data can be included in the third line of evidence, even though the applicability of modeling is much broader. Models are mainly used to verify the long-term efficiency of the MNA and possibly predict the timeframe for the remediation goals to be reached.

1.5 Outline and delimitation of the Ph.D. project

Objectives

The objectives of this Ph.D. project were to identify, apply and evaluate different analytical tools with respect to their potential use as qualitative and possibly quantitative *in situ* indicators of biodegradation in groundwater contaminant plumes.

Approaches

The project was specifically targeted at the *in situ* demonstration of field scale biodegradation, while the general site characterization in terms of e.g. hydrogeology and redox conditions was not an issue, although the data interpretation was necessarily based on knowledge of flow and redox conditions. The main focus was put on three groups of compounds: Petroleum hydrocarbons, phenoxy acid herbicides, and chlorinated compounds.

Relevant methods and tools were identified based on a literature review, and included specific degradation products, stable carbon (and hydrogen) isotope ratios, enantiomeric fractions and compound ratios. On the contrary, e.g. mass flux approaches or molecular *in situ* techniques were not included in the project.

The field sites used for application included two Danish landfills in Vejen [90] and Sjoelund [149], respectively, a former agricultural machinery service on the Danish island Bornholm [**IV**, **VII**], and a former gasoline station in Radsted [102]. Laboratory degradation experiments using groundwater and aquifer material from Sjoelund and Bornholm were also included, as well as the development of new analytical methods or optimization/modification of existing methods in order to measure lower concentrations, other/additional compounds or in complicated matrices.

The evaluation of the different methods/tools were based on the performed field and laboratory investigations and existing literature. The goal was to address their specificity and reliability as qualitative *in situ* indicators of biodegradation and their possible use for quantification of mass reduction, but also to recognize limitations and pitfalls. The evaluation did not refer to the lines of evidence approach. Thus, the general potential of the suggested *in situ* indicators for demonstrating field scale biodegradation were assessed independent of existing protocols or guidelines, while their use as first, second or third lines of evidence was considered unimportant.

2. Identification of attenuation processes and use of compound ratios

2.1 Concentration reduction

For MNA to be considered as the remediation at a contaminated site, obviously the observation of a decrease in contaminant concentration with distance from the source is mandatory. Thus, a clear and meaningful decreasing trend of contaminant concentration downgradient from the source along the groundwater flowpath should be identified.

In order to assess whether a contaminant plume is expanding, stationary, or shrinking, the site history should be reviewed and time series of concentration data should be obtained.

2.2 Temporal variations

Point sources are often assumed to leach contaminants continuously. However, different aspects can be identified, which may potentially cause temporal variations in the source strength, such as heterogeneity of the source zone, fluctuations in the level of the groundwater table, or seasonal variations in the recharge. These conditions will also affect the biodegradation possibly occurring in the contaminant plume, since the availability of both electron donors and electron acceptors also vary due to this incontinuity.

In the plume of hydrocarbons formed downgradient of a residual crude oil body, high temporal variability was observed for the less persistent compounds, while the most stable species showed only little variation [40]. It was argued that the concentration of a given compound is determined by a balance between its rate of dissolution from the residual LNAPL and its rate of removal, where the latter is expected to be more compound-specific than the former. Thus, it was suggested that the temporal variations observed for the more degradable compounds was due to variations in biodegradation rates, which in turn could be caused by recharge introducing pulses of oxygen into the groundwater and thereby affecting the activities and perhaps the composition of the microbial community.

At the Radsted field site [102], a former gasoline station, the residual contamination after removing the underground storage tanks has resulted in the formation of a plume consisting primarily of monoaromatic hydrocarbons. The plume was found to show extensive seasonal variations in shape, extent and concentrations, which seemed to be correlated with groundwater table variations. With a fairly constant release of contaminants in the source area, a higher groundwater flux due to an increased hydraulic gradient would lead to lower BTEX concentrations as a result of an initial dilution.

Thus, time series should be interpreted with care, in order to properly distinguish between seasonal variations and an overall shrinking or expanding plume. It is also important to compare the residence time in the plume with the temporal variation (seasonal or long-term) of the source term, in order to interpret changes along the flowpath correctly.

2.3 Dilution

Since any natural attenuation process can cause a reduction of concentration, the significance of different attenuation processes needs to be evaluated. One important concern is the possibility to confuse biodegradation with dilution.

In order to detect the extent of dilution in the system, a flowline approach using a conservative tracer can be applied. The approach assumes stationarity of the plume and a fairly constant flow velocity in a period corresponding to the transport time. Another important issue is the existence of a suitable tracer, which migrates conservatively and is measurable in significant concentrations after dilution compared to background values. Chloride is a possible candidate as conservative tracer of landfill leachate plumes [17,39,149], since leachate is associated with high concentrations of chloride (150-4500 mg/L [28]; 126-18400 mg/L [11]). The presence of other sources of chloride, e.g. road salt for de-icing purposes or agricultural activities, may at some sites reduce the contrast between leachate-affected groundwater and pristine groundwater [17].

Tritium has also been used as a tracer of landfill leachates based on the fact that landfills from the 1950s to 1970s would have received precipitation enriched in ^{3}H and on the assumption that the rainwater is partially trapped and has a relatively long residence time in the landfill [27,53].

In the absence of a suitable tracer associated with the source, tracer injection experiments can be performed [37]. This involves the injection of a proper compound, e.g. chloride [67] or bromide [5,24,122] in the source zone and subsequent monitoring in the downgradient wells, possibly allowing for the determination of dilution and mean residence times.

The dilution of the leachate plume at the Vejen landfill was significant, resulting in a decrease in chloride concentration of an order of magnitude over a distance of 150 m [**II**]. To assess whether the observed decrease in the concentrations of specific organic compounds was only a result of this dilution, or if biodegradation might be contributing, the concentrations were multiplied by a dilution factor, F, based on chloride concentrations [90]:

$$F = \frac{(C_0 - C_B)}{(C - C_B)} \tag{2.1}$$

where C is the chloride concentration of the groundwater sample, C_0 is the chloride concentration at the border of the landfill, and C_B is the background chloride concentration for the unpolluted aquifer.

Plotting the dilution-corrected concentrations vs. distance (Figure 2.1) revealed that most of the specific organic compounds (alkylbenzenes, alkyl- and chlorophenols [**I**], bicyclo-compounds) were attenuated more than what could be explained by dilution, thus indicating *in situ* biodegradation as a likely process in the landfill leachate plume. Of the specific organic compounds in the leachate plume, only benzene, MCPP and benzylsuccinate (BSA) were not attenuated by other processes than dilution.

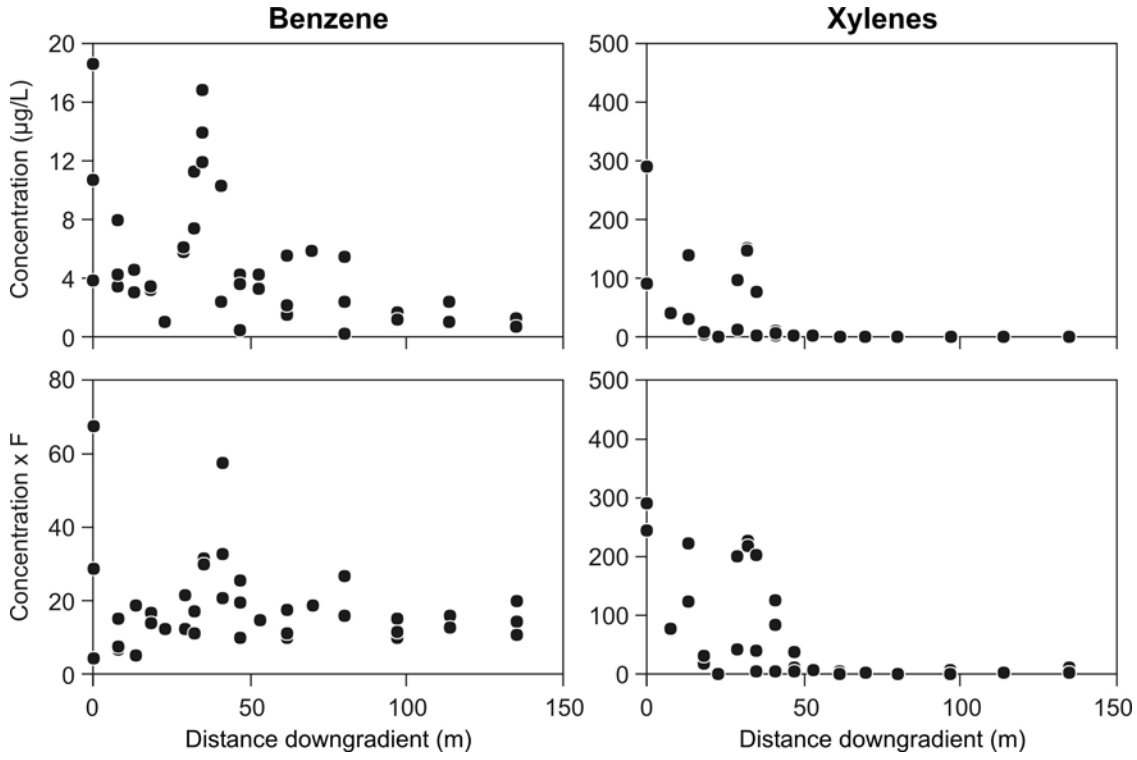

Figure 2.1 Concentrations and dilution-corrected concentrations of benzene and xylenes (*o*, *m/p*) in groundwater downgradient of the Vejen landfill (data from Baun et al. (2003) [**II**])

2.4 Sorption and volatilization by use of compound ratios

To test for the relevance of sorption in the attenuation of a hydrocarbon plume associated with a source of free phase crude oil spilled from a ruptured pipeline, Eganhouse et al. (1996)[40] plotted a series of homologues (benzene/ toluene/ ethylbenzene/ n-propylbenzene or toluene/*o*-xylene/ 2-ethyltoluene/ 2-propyltoluene) as their normalized concentrations versus distance from the source. For each

additional methylen-group added in a homologue series, the K_{ow}-value increases, and thus a decreased mobility in the aquifer is expected if sorption is a dominant attenuation process. However, this is not the observed pattern at the site, and for the latter homology the relationship appears to be exactly the opposite. Large differences in migration are also observed for pairs of isomers, which from their similar K_{ow}-values are expected to be equally retarded by sorption. It is concluded that these observations altogether indicate *in situ* degradation as the plume controlling parameter.

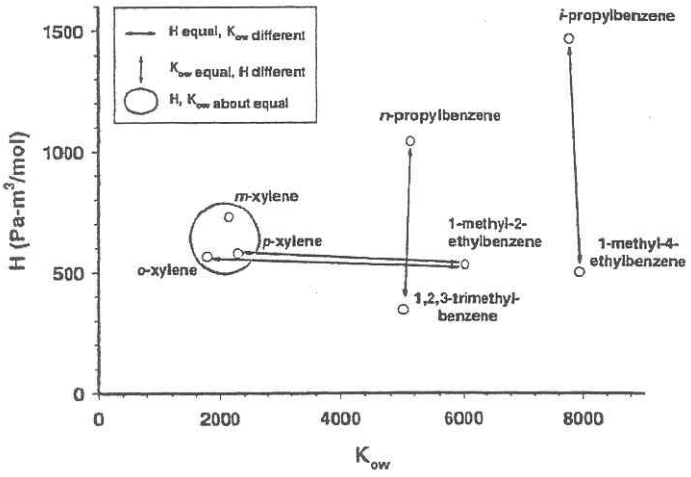

Figure 2.2 Scatterplot showing the distribution of Henry's law constants (H) and octanol-water partition coefficients (K_{ow}) for C2- and C3-benzenes used in the analysis of natural attenuation processes (From Eganhouse et al. [39]. Reprintet with permission of the National Ground Water Association. Copyright 2001).

In a study on the landfill leachate plume downgradient of an unlined municipal landfill, Eganhouse et al. (2001)[39] used alkylbenzenes as process probes for distinguishing different attenuation processes. Dilution was insignificant in the plume as indicated by constant concentrations of chloride, while the compound ratios between different alkylbenzenes were used to demonstrate the relevance of sorption, volatilization as well as degradation as contributors to the observed attenuation of volatile organic compounds (hydrocarbons, oxygen-bearing compounds, and chlorinated hydrocarbons). Thus, different monoaromatic hydrocarbons were paired according to the similarity of their K_{ow}-values and Henry's law constants (Figure 2.2). A constant compound ratio along the flow line for pairs of compounds with similar K_{ow}-values but significantly different Henry's law constants indicated that volatilization was not an important process. In the same way, sorption was shown to be of minor importance by pairing compounds of similar Henry's law constants but

significantly different K_{ow}-values. The xylene compound ratios, on the contrary, did change along the flowline, and since these compounds have rather similar K_{ow}-values as well as Henry's law constants, only degradation (with different first-order rate constants) can be responsible for this change. Thus, using compound ratios it could be concluded that biodegradation was the most important process affecting the fate of volatile organic compounds at this site.

2.5 Degradation by use of compound ratios

The study by Eganhouse et al. (2001)[39] that is described above suggests that several different pairs of compounds (e.g. pairs of isomers or homologues) can possibly be identified, which can be used to verify the *in situ* biodegradation of contaminant plumes in groundwater. For a compound ratio to be useful as an *in situ* indicator of degradation it should be constant everywhere in the field if the two compounds are not degraded, while the degradation of one of the compounds or of both compounds but at different (first-order) degradation rates would change the compound ratio. In other words, all of the other attenuation processes controlling the plume should not affect the compound ratio. Thus, the two compounds should be associated with the same source or the same part of the source in the case of an inhomogeneous source, in order to be identically diluted. Depending on the relevance of the process in the actual system, the compounds should show a similar degree of sorption, volatilization, etc.

A method based on simple mathematical equations was presented by Hansen and Seifert (2000) [57]. Based on the assumptions of the compounds following first-order degradation kinetics, and being equally subjected to non-destructive attenuation processes, an expression of the concomitant changes in the concentrations of the two compounds were deduced. Thus, at any time, t, the concentrations of compound A and B are given by the first-order degradation equations:

$$[A] = [A]_0 \cdot e^{-k_A \cdot t} \tag{2.2}$$

and

$$[B] = [B]_0 \cdot e^{-k_B \cdot t} \tag{2.3}$$

where k_A and k_B are the first-order rate constants, and $[A]_0$ and $[B]_0$ are the initial concentrations of compounds A and B, respectively. The combination of the two equations through the isolation of t, and rearrangement leads to the expression:

$$\ln[B] = \frac{k_B}{k_A} \ln[A] + \left(\ln[B]_0 - \frac{k_B}{k_A} \ln[A]_0 \right) \tag{2.4}$$

From this equation it follows that a plot of the logarithmic concentrations of two compounds with identical physical properties will form a straight line. Thus, the plotting of field concentration data according to eq. 2.4 could be a way to identify field scale biodegradation.

At the Radsted field site [57,102], a former gasoline station, the residual contamination after removing the underground storage tanks has resulted in the formation of a plume consisting primarily of monoaromatic hydrocarbons. The plume is subject to natural attenuation as evidenced by a clear decrease in hydrocarbon concentrations along the flowline. Application of eq. 2.4 to different pairs of alkylbenzenes showed good linear correlations (Table 2.1) [57]. In most cases, the slope was close to 1, indicating that the compounds were degraded at similar first-order rates or that they were not degraded. However, the separation of data according to the redox conditions of the sampling points (aerobic, nitrate-reducing and iron-reducing) revealed some variations. The most significant deviation from a slope of 1 was the slope of 3.05 found for *o*-xylene vs. *m/p*-xylene under iron-reducing conditions (Table 2.1). This indicated that a preferential degradation of *o*-xylene occurred in the iron-reducing part of the plume, and illustrates how compound ratios can be used to demonstrate *in situ* degradation.

Table 2.1 Linear regression of datasets of (ln[A], ln[B]) from the Radsted field site for two compounds, A and B, having approximately similar physical and chemical properties (TMB: trimethylbenzene). The slope corresponds to the ratio, k_B/k_A, between their first-order rate constants according to eq. 2.4 (from Hansen and Seifert (2000) [57]).

Compound		Slope of the regression line for different electron acceptors (correlation coefficient, R^2)			
A	B	Oxygen	Nitrate	Iron	Total
m/p-xylene	*o*-xylene	0.84 (0.90)	0.98 (0.92)	3.05 (0.57)	n.a.
m/p-xylene	ethylbenzene	0.86 (0.58)	1,28 (0.88)	0.82 (0.83)	1.09 (0.91)
1,2,3-TMB	1,2,4-TMB	n.a.	n.a.	n.a.	1.00 (0.86)
1,3,5-TMB	1,2,4-TMB	n.a.	n.a.	n.a.	0.90 (0.67)
1,3,5-TMB	1,2,3-TMB	n.a.	n.a.	n.a.	0.95 (0.72)
m/p-xylene	∑ TMB	n.a.	1.03 (0.98)	0.62 (0.89)	0.91 (0.93)

n.a.: not available

2.6 Application of compound ratios at the Vejen landfill

At the Vejen landfill all of the alkylbenzenes from C1- to C3-benzenes were included in the analysis of hydrocarbons. This provides an opportunity to test the compound ratio approach at an additional (and maybe more complex) site. Baun et al.

(2003) [**II**] showed that all hydrocarbons except benzene were attenuated more than what could be explained from dilution, and from different lines of evidence it was concluded that the majority of this additional attenuation was due to biodegradation. Thus, it would be interesting to learn whether the alkylbenzene compound ratios would support this conclusion.

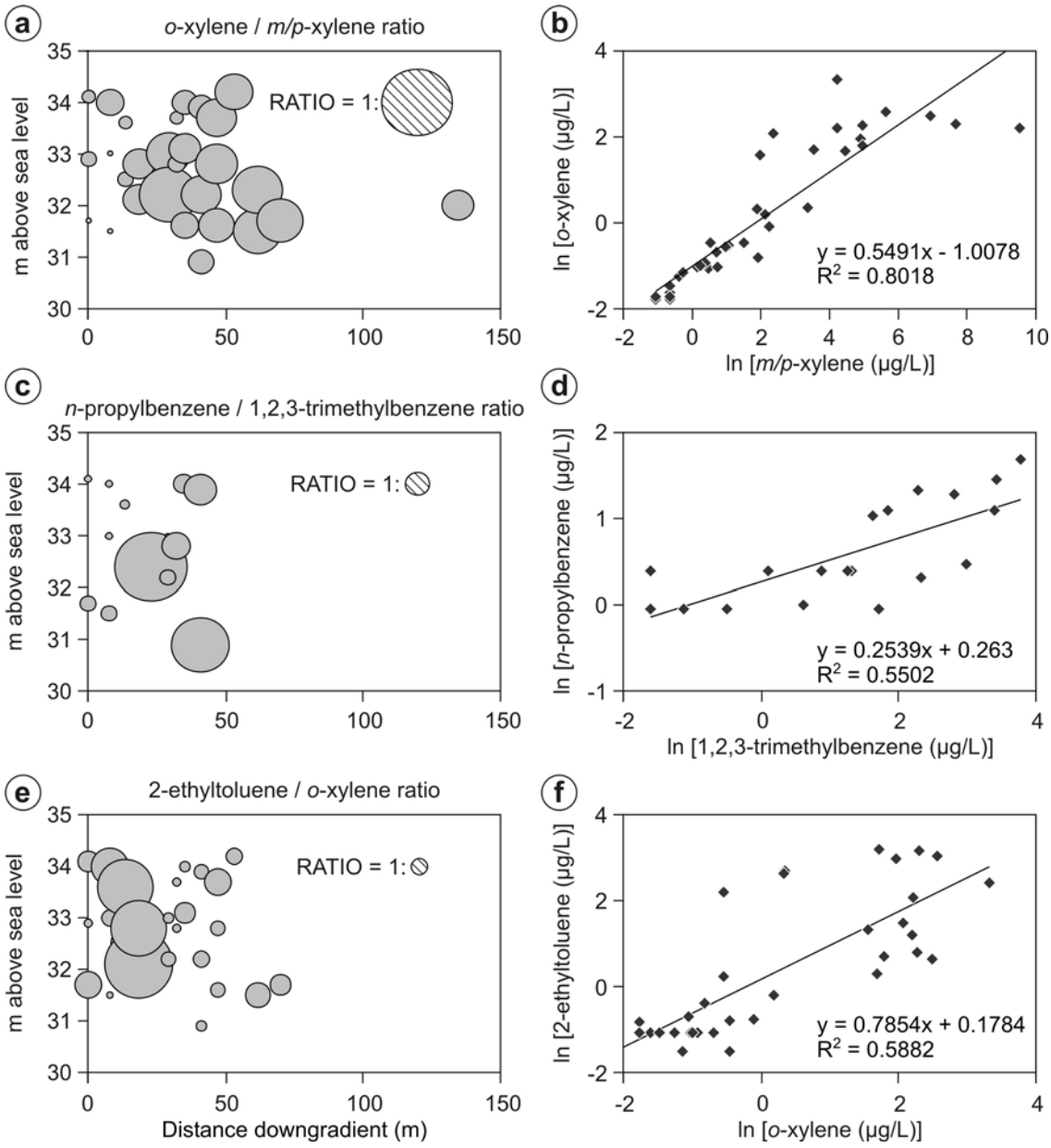

Figure 2.3 Compound ratios in the Vejen landfill leachate plume used for evaluation of natural attenuation processes. Left: Distribution of compound ratios. Right: Double logarithmic plot of compound concentrations (the same dataset as in Baun et al. (2003) [**II]**).

Figure 2.3 shows how some of the same compound ratios applied by Eganhouse et al. (2001) [39] vary in the Vejen leachate plume. The *o*-xylene/*m/p*-xylene ratio (Figure 2.3a) is supposed to be affected only by degradation, and the increase observed in parts of the plume could therefore be taken as indicative of the *m/p*-xylene isomers being degraded at a higher (first-order) rate than *o*-xylene. The plot of the logarithmic concentrations (Figure 2.3b) reveals a significant linear correlation, and again a higher degradation rate for *m/p*-xylene compared to *o*-xylene is indicated by the slope being less than unity. Thus, the *o*-xylene/*m/p*-xylene ratio supports and strengthens the original conclusion of biodegradation as an important attenuation process, which demonstrates that the compound ratio approach can be feasible even in a complex flow system.

The *n*-propylbenzene/1,2,3-trimethylbenzene and *i*-propylbenzene/4-ethyltoluene ratios should decrease if volatilization is an important attenuation process. The former actually do show a decreasing trend (Figure 2.3c-d), while the latter shows no systematic variation (data not shown). Volatilization alone cannot explain this behavior, but on the other hand the compound ratios do not prove the absence of volatilization as a significant factor for the attenuation of alkylbenzenes.

The 2-ethyltoluene/*o*-xylene ratio was used by Eganhouse et al. (2001)[39] to test the significance of sorption. At Vejen, this ratio appears to increase from 0m to 20m, decrease from 20m to 30-40m, and then increase again (Figure 2.3e). This development suggests that sorption is significant in an area of the plume from 20-40m downgradient of the landfill. This is consistent with the appearance of a low-permeable zone about 25-30m downgradient [**II**]. The attenuation in other parts of the plume seems to be dominated by degradation, with *o*-xylene being more easily degraded than 2-ethyltoluene. The linear fit in Figure 2.3f indicates a weak decrease, which is what should be expected from sorption. The opposite trends observed for the 2-ethyltoluene/*o*-xylene ratio in different parts of the plume are probably offsetting each other in this kind of plot. This illustrates that the application of the logarithmic plot fails for compounds with different physical-chemical properties. Indeed, the similarity of physical-chemical properties was a prerequisite for the validity of eq. 2.4.

Several other pairs of compounds at the Vejen field site of e.g. alkylbenzenes, alkylphenols, bicyclo-compounds and methylnaphthalens were also plotted (ln[B] vs ln[A]) (data not shown), the highest linear correlation being observed for the pairs of highest structural similarity (e.g. trimethylbenzenes), while a linear correlation was completely absent for compound pairs with obvious structural differences such as e.g. camphor/2-ethyltoluene. An alternative explanation for the lack of a correlation between the concentrations of two compounds (apart from the compounds having too different physical-chemical properties) could be that they are leaching from different parts of the landfill, and therefore they follow different flowpaths in the plume.

2.7 Discussion

Confusion of temporal variations and dilution with biodegradation during site assessment are clearly among the obvious pitfalls. Time series should therefore be planned carefully, and the possible use of a conservative tracer (naturally occurring or injected) should be considered. However, tracer tests may in complex systems lead to ambiguous results.

The use of compound ratios as illustrated by the use of alkylbenzenes in different case studies [39,40,57] is very simple in the sense that it implies no additional analyses. The approach of pairing/grouping structurally similar compounds with similar or systematically different K_{ow}-values and Henry's law constants can easily be transferred to other classes of compounds. Whether the compound ratios are easy to interpret depends on the complexity of the site.

Overall, compound ratios can be useful and easily applied indicators of *in situ* degradation, but interpretation should be done with solicitude, especially at sites characterized by a complex source and flow pattern. It should be carefully evaluated whether a pair of compounds adequately fulfills the criteria of being associated with the same source and being equally subjected to physical processes.

3. Redox indicators

3.1 Degradation of bulk contamination

The metabolic degradation of organic matter involves the oxidation of an organic compound (electron donor, ED) and a corresponding reduction of an electron acceptor (EA). Depending on the used electron acceptor the microorganisms gain different amounts of energy from the degradation. In groundwater, a series of electron acceptors of decreasing reduction potential is available: $O_2 > NO_3^- > Mn(IV) > Fe(III) > SO_4^{2-} > CO_2$. When a point source contamination is subject to significant biodegradation, the supply of electron acceptors cannot counterbalance the rate of consumption. This results in the successive depletion of electron acceptors and accumulation of reduced species such as Mn(II), Fe(II), and S(-II), and the development of redox zones going from mostly anaerobic in the core of the plume to aerobic at the plume fringes [28,157].

When the concentration decrease of a contaminant plume is associated with a corresponding occurrence of redox zones, it might be indicative of biodegradation. However, care should be taken not to confuse cause and effect.

Petroleum hydrocarbons constitute a class of compounds that are generally degraded under all redox conditions [21]. The compounds serve as primary substrate for degrading microorganisms, and can be directly linked to the consumption of electron acceptors. Thus, the occurrence of redox zones at hydrocarbon contaminated sites is a valid *in situ* indicator of biodegradation, and is the most commonly applied second line of evidence in MNA of petroleum hydrocarbons [21,114,157].

At the Radsted field site [102], a former gasoline station, the residual contamination after removing the underground storage tanks has resulted in the formation of a plume consisting primarily of monoaromatic hydrocarbons. The plume is subject to natural attenuation as evidenced by a clear decrease in hydrocarbon concentrations along the flowline. Measurement of the redox parameters revealed the occurrence of a sequence of redox zones concomitant to the contaminant plume (Figure 3.1), with iron- and manganese-reducing conditions in the core of the plume, nitrate-reducing conditions at the plume fringes and downgradient of the plume, while the unaffected groundwater was aerobic. With that, it was rendered probable that substantial biodegradation occurred at the site.

3.2 Degradation of specific compounds

While the redox parameters can serve as an *in situ* indicator of the biodegradation of petroleum hydrocarbons, this is not the case with another major class of groundwater contaminants: The chlorinated solvents. These highly chlorinated aliphatic hydrocarbons are known as recalcitrant under aerobic conditions, but can be degraded under strictly anaerobic conditions by reductive dechlorination [156]. However, this

degradation process is not coupled to the consumption of the above-mentioned electron acceptors (O_2, NO_3^-, etc.). Rather, the chlorinated compounds themselves act as electron acceptors, meaning that another source of organic carbon must be present, which can serve as electron donor in the redox reaction. Thus, the degradability of the chlorinated solvents is extensively related to the redox conditions, but their degradation will not result in the development of sequential redox zones. Thus, the causal relationship between contaminant degradation and occurrence of redox zones for chlorinated solvents is quite the opposite to that of petroleum hydrocarbons.

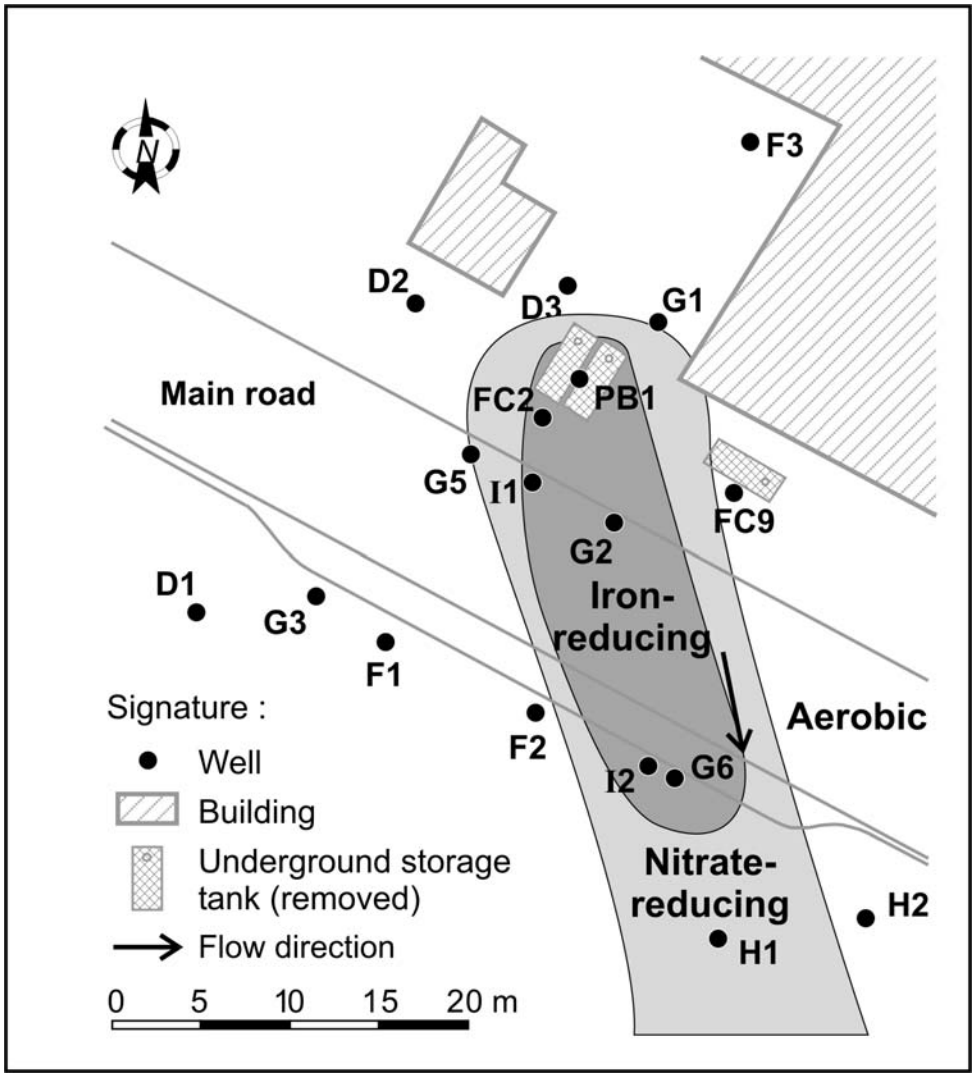

Figure 3.1 Map of the Radsted field site, showing the redox conditions developed downgradient of the removed underground storage tanks (from Ledin et al. (2005) [**V**]).

It should be realized that the distribution of redox parameters as *in situ* indicators of biodegradation only relates to the bulk contamination. A petroleum hydrocarbon contamination for instance consists of multiple compounds. The occurrence of redox zones only indicates that substantial biodegradation occurs, but not that every single compound is being degraded. This implies that some critical compound, making up only a minor part of the total hydrocarbon load, but still present at concentrations of environmental concern, might escape the attenuation processes and eventually reach downgradient receptors.

A typical example of such a critical compound in relation to petroleum hydrocarbon contaminations is the presence of MTBE (methyl-*tert*-butylether), the most commonly used fuel oxygenate. MTBE is characterized by high mobility and low degradability compared to e.g. BTEX, which means that while BTEX plumes may stabilize on average within 100 m of the source [137], MTBE may continue to migrate further downgradient. Thus, the expected redox zones are still created due to the degradation of BTEX, but the possible degradation of MTBE remains to be demonstrated.

Landfill leachate plumes are also associated with redox plumes due to the degradation of the high concentrations of dissolved organic carbon (DOC) generally leaching from landfills [11,28]. Thus, the general degradation of the bulk organic carbon can be demonstrated through a characterization of redox conditions, but analogously to the MTBE example above, different specific organic compounds may be present, which are not degraded in the plume core. The MCPP and benzene in the Vejen landfill leachate plume are examples of this (Baun et al. (2003) [**II**], Figure 4). These potentially critical compounds migrate further downgradient where they eventually may degrade under more oxidized redox conditions. Still, alternative tools are necessary in order to demonstrate their possible degradation.

3.3 Quantification of biodegradation and mass balances

The existence of redox zones in relation to a plume of contaminants such as petroleum hydrocarbons can be used as a qualitative as well as a quantitative indicator of biodegradation of the bulk contamination. The consumption of electron acceptors can be determined based on observed changes along a flowline in the concentrations of electron acceptors or reduced byproducts (e.g. Fe(II), Mn(II)). The corresponding amount of an organic compound degraded can then be calculated. The result can be validated by setting up mass balances [16,72,144].

Thornton et al. (1998) [144] calculated electron balances for contamination plumes at two sites, one plume consisting of ammonium and some phenolic compounds, and the other consisting primarily of coal-tar compounds (alkylphenols, pyridins, and BTEX). At the first site, the electron balance showed that the disappearance of ammonium (by nitrification) agreed well with the observed consumption of electron acceptors, while at the other site, the consumption of electron donor (the organic

contaminants) was much higher than the consumption of electron acceptors. However, the ED consumption in terms of produced dissolved inorganic carbon (DIC), agreed relatively well with the EA consumption. This inconsistency at this site could be due to the under-estimation of EA consumption or over-estimation of ED consumption. Suggested reasons for the former were the precipitation of iron(II)sulphide, and a limited availability of groundwater-born electron acceptors such as oxygen and nitrate, which led to total depletion in the core of the plume. Reasons for overestimation of ED consumption could be that the contaminants are not completely mineralized, or that they are partly attenuated by other processes. Based on the electron balances, the authors concluded that the contaminant plume was probably expanding.

As suggested above, the production of DIC instead of the consumption of organic carbon can be compared with the consumption of electron acceptors. The complete mineralization of organic compounds to CO_2 may lead not only to changes in DIC, but also in alkalinity, depending on the redox conditions. Thus, by measuring the alkalinity a second mass balance can be added [16,72,73], which may strengthen the argumentation. The precipitation or dissolution of solid carbonates may buffer the changes in DIC, alkalinity and pH, why e.g. changes in Ca^{2+} and Mg^{2+} should also be included in the mass balances [16]. While the mineralization of organic compounds always leads to the production of CO_2, which in groundwater systems will add to the DIC, only mineralization under certain redox conditions leads to a change in alkalinity.

At a hydrocarbon-contaminated site in Menziken (Switzerland) [72], the expected change in alkalinity based on EA consumption agreed well with the observed change in alkalinity, while the expected change in DIC was under-estimated compared to the observed change in DIC. Thus, it was inferred that the disagreement was connected to alkalinity-neutral processes, which could be aerobic or methanogenic degradation. The distribution of methane in the plume suggested that methanogenesis occurred and that volatilization of methane was responsible for the under-estimation of methane production.

Similarly, for a BTEX plume at a former industrial site in Drejøgade, Copenhagen [16], the observed change in DIC, but not the change in alkalinity, was much higher than expected from the observed EA consumption, indicating that aerobic respiration and/or methanogenesis was under-estimated. Thus, it was concluded that gas transport (oxygen, methane) between the unsaturated zone and the groundwater was important at the site.

3.4 Discussion

The measurement of redox parameters at contaminated sites is compulsory, since a thorough understanding of the redox conditions is a prerequisite for interpreting any other evidence of biodegradation.

The oxidation of point source contaminants can cause a depletion of electron acceptors, leading to a characteristic pattern of successive redox zones, which is indicative of the oxidative degradation of bulk contaminant. This makes redox parameters suitable as *in situ* indicators of biodegradation when it comes to e.g. hydrocarbon contaminations (in the absence of oxygenates such as MTBE), while reductive degradation cannot be demonstrated in this way. Also, the EA consumption associated with the degradation of specific compounds in low concentrations compared to the total amount of organic carbon cannot be distinguished from bulk degradation, and therefore cannot be demonstrated.

The EA consumption can be quantified and converted to contaminant consumption, thus providing an estimate of the biodegradation at the site. Further understanding of the pollutant attenuation at the specific site can be achieved through the setup of mass balances, which can also serve as a valuable tool for validation of the obtained estimate of the biodegradation.

4. Degradation products

4.1 Biodegradation of organic compounds

Most microorganisms will biodegrade organic compounds in metabolic processes where they gain both carbon and energy. In this way, the organic compounds are oxidized and eventually transformed into CO_2. Different amounts of energy are obtained from the oxidation depending on which electron acceptor is used, the highest energy yield being obtained from the use of oxygen. However, some organic compounds are not (or not always) metabolically degraded, but may be degraded by co-metabolic oxidation or reduction.

4.1.1 Specificity of metabolites

Apart from the ultimate degradation product, CO_2, various intermediate metabolites have been identified for different organic contaminants. Some of these metabolites have been proposed for use as *in situ* indicators of biodegradation.

CO_2 is completely unspecific, as it is the degradation product from the complete oxidation of any organic compound. Furthermore, a significant background value and the possible shifts in the equilibria of the carbonate system complicate the use of CO_2 as an *in situ* indicator of biodegradation. Intermediate degradation products, dead-end products, and end-products of reductive degradation processes can be more or less specific. Beller (2000) [13] formulated four characteristics of a metabolite, which should be fulfilled for the metabolite to be an ideal indicator of *in situ* alkylbenzene metabolism (Table 4.1). These characteristics can also be applied to metabolites of other compounds in order to evaluate their suitability as *in situ* indicators of biodegradation.

Some metabolites are unequivocally related to the degradation of a single compound, while others may be derived from a variety of organic compounds or may have other sources than degradation. Generally, metabolites are more specific, when a large portion of the parent compound structure is still retained, which is valid for the first degradation steps. At more advanced stages of biodegradation, the metabolites are less specific for the degradation of a single compound, but tend to become more characteristic of bulk degradation of the present organic compounds in general.

Table 4.1 Ideal characteristics for indicators of *in situ* degradation (Modified after Beller et al. (2000) [13].

Characteristic of metabolite	Significance
An unequivocal and unique biochemical relationship to the parent compound	When found in groundwater, the compound can be definitively related to the metabolism of a specific compound
No commercial or industrial uses	When found in groundwater, the compound can be definitively related to metabolism and not to anthropogenic releases of the compound ifself
Biological and chemical stability	Stability increases the probability of detecting the compound in groundwater
Generation as an intermediate of mineralization rather than as a product of cometabolism	The goal of bioremediation is mineralization to innocuous products, not transformation to potentially toxic metabolites

4.1.2 Impurities

The demand for no commercial or industrial uses should be paid special concern, because synthetic compounds for commercial or industrial use are manufactured as technical grade compounds. Thus, the compounds may contain impurities, some of which could be identical to known or putative metabolites associated with the degradation of that compound, while they may not have a commercial or industrial use themselves [**IV**]. The impurities do not necessarily make up a large fraction of the technical grade compound, although for instance technical grade HCH (1,2,3,4,5,6-hexachlorocyclohexane, an insecticide) contains only 15% of the active ingredient, γ-HCH, while the last 85% are impurities (other conformational isomers of HCH) without insecticidal activity [26]. But even small amounts of impurities can be significant and measurable in contaminated groundwater.

The reason why metabolites sometimes can be identical to impurities in the synthetic compound undergoing degradation could be that small amounts of a reactant during synthesis is preserved in the final product as an impurity. Then, if the degradation pathway or part of it is simply the reversed synthesis reaction, the impurity will be produced as a degradation product and added to the original amount of impurity. Thus, the presence of compounds, which are potential degradation products as well as impurities, should be interpreted with solicitude.

4.1.3 Stability and further degradability of metabolites

In order to detect a metabolite in the groundwater, the metabolite obviously needs to have a sufficient biological and chemical stability. But in addition to this, a prerequisite for metabolites to serve as indicators is that they are released to the

extracellular medium such that they could be expected to occur in contaminated groundwater [13].

However, the metabolite should not be too stable, since the accumulation of dead-end metabolites is not feasible for bioremediation of contaminated groundwater. Some potential indicator metabolites are transient intermediates in some cases, but dead-end metabolites in other cases, depending on e.g. redox conditions and the microbial community. This emphasizes the need for a careful interpretation of the occurrence of metabolites in terms of concentrations and distribution in the contaminant plume.

4.2 Aromatic hydrocarbons

4.2.1 Suggested indicator metabolites

A range of organic acids can be produced from the degradation of hydrocarbons, which could be considered as *in situ* indicators of biodegradation, such as benzoate and methylbenzoates [34,115], benzylsuccinate, E-phenylitaconate, and their methyl homologues [14], and short-chain aliphatic acids [33]. Because aromatic acids persist in an anaerobic plume of hydrocarbon contaminants longer than alicyclic or aliphatic acids, aromatic acids have been suggested as the most ideal *in situ* indicators of aromatic hydrocarbon biodegradation [13,51,107]. Other possible metabolites of aromatic hydrocarbons are aromatic alcohols and aldehydes, which were also proposed to serve as indicators [49].

Recently, Griebler et al. (2004) [51] summarized the proven and suggested pathways and metabolites for the anaerobic degradation of BTEX compounds (Figure 4.1) and naphthalenes (Figure 4.2), primarily through the fumarate addition pathway, which involves the initial addition of a methyl or methylene carbon to the double bond of fumarate. This pathway was first observed for the anaerobic degradation of toluene [15]. The majority of these metabolites (i.e. benzylsuccinates, E-phenylitaconates, and benzoates) were evaluated by Beller (2000) [13] in terms of their suitability as indicators of *in situ* anaerobic alkylbenzene metabolism.

4.2.2 Benzylsuccinates and E-phenylitaconates

It was found that the benzylsuccinates and E-phenylitaconates fulfilled the four criteria (Table 4.1) the best, although the methyl homologues may in some cases be dead-end metabolites, depending on the bacteria involved. However, this is not necessarily of primary concern under field conditions, since compounds generated as dead-end metabolites by a given bacterial species could serve as substrate for other bacterial species and ultimately could be mineralized by the indigenous bacterial

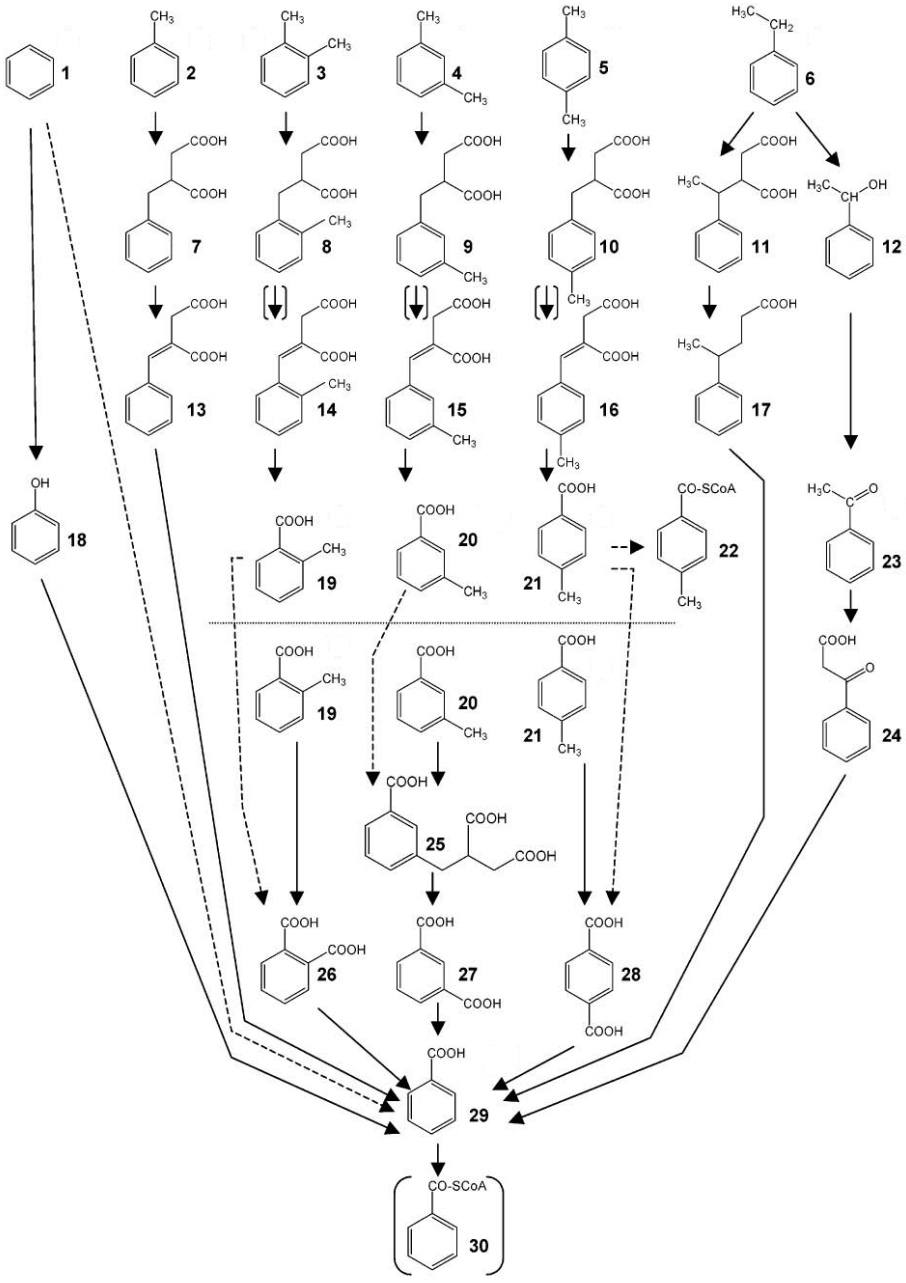

Figure 4.1 Proposed pathways and metabolites for the anaerobic degradation of BTEX compounds: 1, benzene; 2, toluene; 3, o-xylene; 4, m-xylene; 5, p-xylene; 6, ethylbenzene; 7, benzylsuccinic acid; 8, 2-methylbenzylsuccinic acid; 9, 3-methylbenzylsuccinic acid; 10, 4-methylbenzylsuccinic acid; 11, α-methylsuccinic acid; 12, 1-phenylethanol; 13, phenylitaconic acid; 14, 2-methylphenylitaconic acid; 15, 3-methylphenylitaconic acid; 16, 4-methylphenylitaconic acid; 17, 4-phenylpentanoic acid; 18, phenol; 19, o-toluic acid; 20, m-toluic acid; 21, p-toluic acid; 22, 4-methylbenzoyl-CoA representative for all methylbenzoyl-CoA isomers; 23, acetophenone; 24, benzoylacetic acid; 25, 3-carboxybenzylsuccinic acid; 26, phthalic acid; 27, isophthalic acid; 28, terephthalic acid; 29, benzoic acid; 30, benzoyl-CoA. Solid arrows indicate proven pathways, whereas dashed arrows indicate suggested transformation steps. The anaerobic transformation of xylene isomers 3-5 in some cases was observed to be co-metabolic with the corresponding methylbenzylsuccinic acids 8-10 as dead-end metabolites. The further degradation is therefore not obligatory (arrows in brackets) (Reprintet with permission from [51]. Copyright 2004 American Chemical Society).

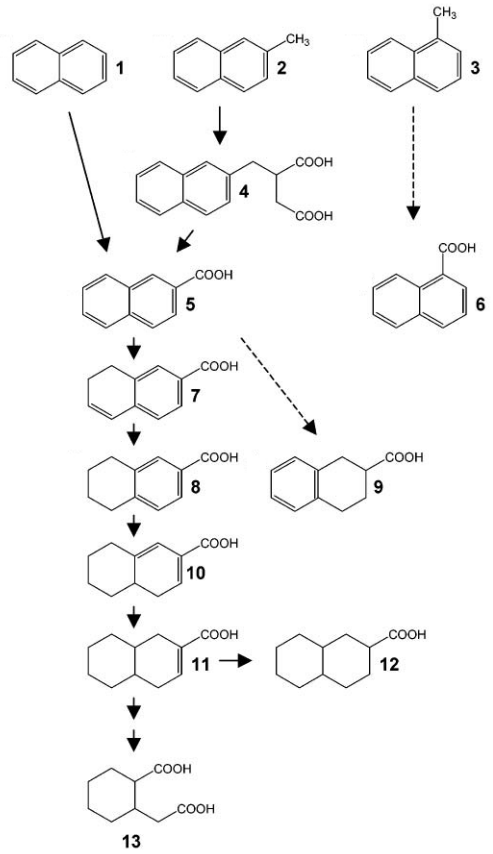

Figure 4.2 Proposed pathways and metabolites for the anaerobic degradation of naphthalenes: 1, naphthalene; 2, 2-methylnaphthalene; 3, 1-methylnaphthalene; 4, naphthyl-2-methylsuccinic acid; 5, 2-naphthoic acid; 6, 1-naphthoic acid; 7, 7,8-dihydro-2-naphthoic acid; 8, 5,6,7,8-tetrahydro-2-naphthoic acid; 9, 1,2,3,4-tetrahydro-2-naphthoic acid; 10, 5,6,7,8,9,10-hexahydro-2-naphthoic acid; 11, 1,4,5,6,7,8,9,10-octahydro-2-naphthoic acid; 12, decahydro-2-naphthoic acid; 13, cis-2-carboxylcyclohexylacetic acid (Reprintet with permission from [51]. Copyright 2004 American Chemical Society).

consortia. On the other hand, it was noted that most of the benzylsuccinates and E-phenylitaconates are not commercially available as standards, which could potentially limit their use as *in situ* indicators. This in turn, convincingly illustrates that the compounds truly have no other sources than degradation. A controlled release experiment of BTEX (excl. *p*-xylene) demonstrated the transformation of toluene, *o*-xylene and *m*-xylene to the corresponding succinate and itaconate derivatives [14]. However, in contrast to the benzylsuccinates [11,13,41,51,94,117], the occurrence of E-phenylitaconates has not been demonstrated at contaminated field sites. Either this is due to a limited number of target metabolites being searched for in the field investigations, or the E-phenylitaconates are immediately further degraded and

therefore never exceed the method detection limits. The latter would make the suitability of E-phenylitaconates questionable as indicator metabolites.

Succinate derivatives of other compounds would also be expected to conform relatively well to the ideal characteristics listed in Table 4.1. Thus, the degradation of 2-methylnaphthalene in microcosms under sulphate-reducing conditions lead to the formation of naphthyl-2-methylsuccinate and naphthylitaconate [6], of which naphthyl-2-methylsuccinate, but not naphthylitaconate, was identified in groundwater from a former gas work site. At a jet fuel contaminated site, the succinate derivatives of several alkylbenzenes ranging from C2- to C6-benzenes were tentatively identified based on their mass spectra [94]. Laboratory experiments have also shown that compounds other than aromatic hydrocarbons can be degraded by the fumarate addition pathway, such as dodecane [79], m-toluic acid (which itself is a metabolite of *m*-xylene) [41], and *p*-cresol [103]. Gieg and Suflita (2002) [50] detected low µg/L levels of the succinate derivatives of different *n*-alkanes in groundwater from different petroleum-impacted aquifers, while the potential occurrence in contaminated groundwater of the succinate derivatives of compounds other than hydrocarbons is yet to be investigated.

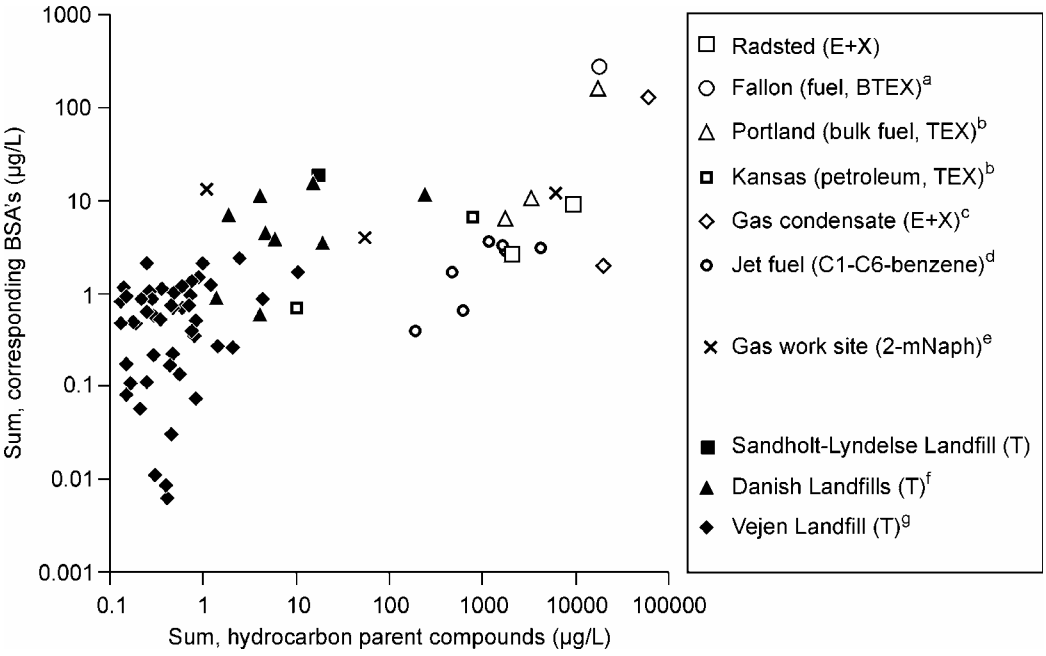

Figure 4.3 Corresponding concentrations of benzylsuccinates (BSA's) and parent hydrocarbons in field investigations (from Ledin et al. (2005) [**V**]).

The sum of concentrations of the benzylsuccinates observed at various field sites were found to show an overall correlation with the concentrations of the hydrocarbon parent compounds (Figure 4.3). For hydrocarbon-contaminated sites the metabolite concentrations were appr. 3 orders of magnitude lower than the parent compound concentrations. At landfills only benzylsuccinate, but not the methyl homologues, were measured, which generally occurred in concentrations within an order of magnitude of the (low µg/L) toluene concentrations. It was speculated, that this discrepancy could be due to the benzylsuccinate metabolite being more persistent, or the fumarate addition pathway being more pronounced in landfill leachate and leachate-affected groundwater compared to hydrocarbon-contaminated groundwater. Persistence of benzylsuccinate(s) within a contamination plume was observed for the leachate plume at the Vejen Landfill [**II**], and for a jet fuel contamination plume at a former military airport in Germany [94], while the concentrations of alkylbenzenes were declining. This resulted in an increasing metabolite/parent compound ratio with decreasing parent compound concentration (Figure 4.4), which as a compound ratio indicates the preferential removal of the alkylbenzene parent compounds. Since the benzylsuccinate concentrations were rather similar at the two sites (low µg/L level), the data points from both sites seem to follow the same trend line. However, at other sites the benzylsuccinate concentrations showed apparently random temporal and spatial variations [41,51]. Importantly, the benzylsuccinates were only detected within (or just downgradient [94]) the contaminated areas [41,94] in the field investigations described so far, which indicates that despite the possible persistence within a plume, eventually the benzylsuccinates are further degraded.

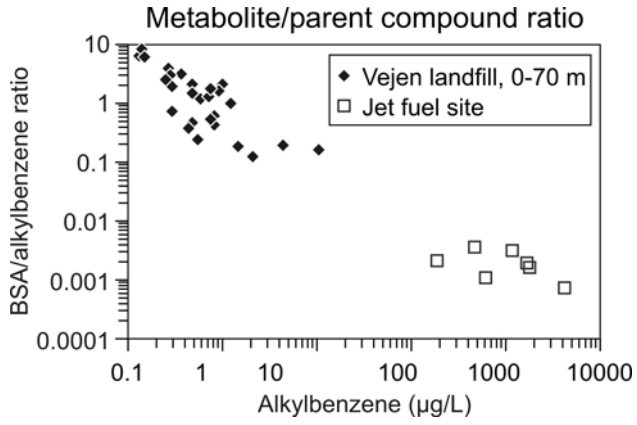

Figure 4.4 The metabolite/parent compound ratio (succinate derivative/ alkylbenzene) as a function of parent compound concentration at the Vejen landfill [**II**] and a jet fuel site [94].

4.2.3 Benzoates

Benzoates were found to conform moderately well to the four ideal characteristics of metabolic indicators cited in Table 4.1 [13], and they have been observed at a variety of hydrocarbon-contaminated field sites [13,41,49,51,94,107]. Three of the characteristics (a unique biochemical relationship to a parent hydrocarbon, no commercial or industrial uses, and biological and chemical stability) apply poorly to benzoate, but fairly well to the more substituted alkylbenzoates. Namocatcat et al. (2003) [107] specifically suggested the use of trimethylbenzoates as key signature metabolites because of their unequivocal biochemical relationship to their parent hydrocarbons (tetramethylbenzenes), and because they were the dominant metabolic intermediates in a jet fuel contaminated aquifer, even though toluene, xylenes, ethylbenzenes and trimethylbenzenes were the major hydrocarbon components, while tetramethylbenzenes comprised only a minor fraction. Still the trimethylbenzoates were not detected neither upgradient nor downgradient of the plume, which points to their further degradation.

In contrast to the benzylsuccinates, which are only formed under anaerobic conditions, benzoates might also be intermediates of aerobic alkylbenzene degradation [134]. Thus, while the occurrence of benzylsuccinates in contaminated groundwater indicates anaerobic degradation of aromatic hydrocarbons, the benzoates only indicate the degradation, but not whether it occurred under anaerobic or aerobic conditions. The aerobic alkylbenzene metabolism could be a possible explanation for finding benzoates but not benzylsuccinate in some hydrocarbon contaminated groundwater, since the infiltration of dissolved oxygen into the anaerobic hydrocarbon plume may result in the creation of fringe zones, where aerobic degradation prevails [21]. Another reason could be that the benzoates occur in higher concentrations (e.g. by 1-2 orders of magnitude [94]). As with the benzylsuccinates, the benzoates may show persistence within a contaminant plume [94], but also substantial temporal and spatial variations [107].

4.2.4 Discussion

In general, benzylsuccinates and E-phenylitaconates are superior to benzoates in terms of their very high specificity to their parent hydrocarbons and their lack of commercial and industrial sources. They are also unequivocally associated with the degradation under anaerobic conditions. As to the chemical and biological stability, the E-phenylitaconates might be too labile, since their occurrence at real contaminated sites are not reported in field investigations so far, while the benzylsuccinates and the benzoates (especially the more substituted alkylbenzoates) can occur in significant concentrations. Although some of the compounds might be dead-end metabolites [13]

or tend to accumulate within a contamination plume [94,107], they are not found in unaffected groundwater, indicating their overall degradation at the field scale.

Overall, the field investigations collectively points to the suitability of benzylsuccinates and benzoates as qualitative *in situ* indicators of biodegradation. On the contrary, the field studies do not unequivocally support a quantitative approach based on these metabolites. This emphasizes the need for more field investigations, preferably including a larger range of target analytes, since the different metabolites included in different studies complicates an interpretation between field sites.

4.3 Chlorinated compounds

4.3.1 Reductive dechlorination

The presence of chlorine atoms in an organic compound generally makes the compound more resistant to biodegradation by oxidative pathways, and more chlorine atoms increase the persistence [54]. However, under strictly anaerobic conditions especially the highly chlorinated compounds can undergo reductive dechlorination [68]. In this process a chlorine substituent is removed from the molecule (and released as chloride) with concurrent addition of electrons.

If complete dechlorination is feasible, a series of sequentially more dechlorinated compounds will be formed as degradation products. But since the lower chlorinated compounds are less amenable to reductive dechlorination, there is a risk that these compounds may accumulate at some sites.

4.3.2 Chlorinated ethenes

The chlorinated solvents, perchloroethylene (PCE) and trichloroethylene (TCE), are among the most common groundwater contaminants. Their degradation pathway by reductive dechlorination is well-known, and goes through dichloroethene (DCE, primarily *cis*-1,2-DCE), vinylchloride (VC) to ethene and ethane [156]. A sequential formation of dechlorination products has been observed in several microcosm experiments [44,71,91].

With reference in the four characteristics of an ideal *in situ* indicator of biodegradation (Table 4.1), which were set up for the metabolism of alkylbenzenes [13], the degradation products from the reductive dechlorination conform well to the requirement of a unique biochemical relationship to the parent compound, and biological and chemical stability. However, the demand for no commercial or industrial uses is not fulfilled, since 1,2-DCE (mainly *trans*) is among other things a solvent itself, and VC and ethene are for instance used in the manufacturing of polymers [154]. Furthermore, the dechlorination products are impurities in TCE and PCE (typically <1%) probably due to incomplete chlorination during the synthesis.

The generation of the metabolites as dead-end products can also be a problem at some sites, causing accumulation of *cis*-1,2-DCE or VC.

Still, the concentrations and the distribution of dechlorination products in a contaminant plume have proven a powerful tool for the evaluation of *in situ* biodegradation at various field sites. Often the dechlorination products occur in concentrations that by far exceed the concentrations, which can be expected from the impurity content of the parent compounds. Their possible origin from other industrial uses at the specific site may often be uncovered by a review of site history. Only at some multiple contaminant sites, such as landfills, this will not be possible.

4.3.3 Other chlorinated compounds

Several other aliphatic and aromatic chlorinated compounds (e.g. chlorinated ethanes and methanes, chlorobenzenes, chlorinated biphenyls, pentachlorophenol etc.) have been shown to undergo reductive dechlorination, resulting in the formation of less chlorinated daughter products [139], which like the dechlorination products of PCE/TCE, all conform relatively well to the ideal indicator characteristics of a unique biochemical relationship to the parent compound, biological and chemical stability as well as their being intermediates in the degradation pathway. A selection of the daughter products may have specific commercial or industrial sources, whereas most of them are likely to occur as impurities in the parent compounds.

Pentachlorophenol (PCP) has been widely used as a pesticide and a wood preservative. Technical grade PCP contains large amounts of tetrachlorophenols and smaller amounts of tri-, di- and monochlorophenols [147]. Upon the biodegradation the dechlorination occur more easily at the *o*- and *p*-positions, why the intermediate metabolite 3,5-dichlorophenol may tend to accumulate [154]. Small amounts may, dependent on how PCP was synthesized, be present as an impurity in PCP, but the compound has no commercial or industrial uses. The detection of 3,5-dichlorophenol, but absence of higher chlorinated phenols, in the leachate plume at the Vejen landfill site [**II**], as well as in leachate from other landfills [11] indicate that PCP was deposited in the landfills, but effectively attenuated either by sorption ($\log K_{ow}$ = 4.07/5.01 [19]) or by degradation through anaerobic dechlorination [11].

4.3.4 Field application

The classical pattern observed at sites, where natural attenuation of chlorinated solvents has been reported [82,91,156] is the dechlorination product plumes successively offset downgradient from the higher chlorinated parent compounds. Thus, the TCE plume would extend beyond the PCE plume; the DCE plume would extend beyond the TCE plume, and so on. A substantial increase in chloride concentration with distance from the source can also be indicative of dechlorination.

In order to facilitate the interpretation of the distribution of parent and daughter compounds, a dechlorination index can be calculated, which is a quantitative method to determine the degree of dechlorination [63]. If dechlorination occurs, the dechlorination index should increase with distance from the source. Adding up the molar concentrations of parent and daughter compounds, and then use the mole fractions instead of the concentrations may also facilitate the identification of a progressing dechlorination process [76,87].

The prevalence of anaerobic conditions is mandatory for a complete dechlorination to occur. Since the anaerobic conditions are not created by the degradation of chlorinated solvents, the feasibility of anaerobic dechlorination depends on the presence of another source of carbon, which can act as an electron donor. A number of chlorinated solvent contaminations occur at e.g. industrial sites, where point sources of other contaminants can be found as well. Degradation of these contaminants, e.g. BTEX [63,160], non-chlorinated organic solvents such as methanol [91], or organic waste [82] can possibly create the anaerobic conditions necessary for anaerobic dechlorination. Yet, the extension of the strictly anaerobic area is at some sites not sufficient for the dechlorination to go to completion. The partly dechlorinated ethenes and even residual parent compounds may then potentially migrate to downgradient receptors without further transformation [63]. However, the less chlorinated daughter products are more prone to oxidative degradation than their highly chlorinated parent compounds, why they might eventually be degraded under aerobic conditions. Indeed, a mixture of anaerobic and aerobic degradation processes were found to efficiently attenuate plumes of chlorinated solvents at some sites [76,160].

4.3.5 Discussion

The presence of strictly anaerobic conditions is a prerequisite for the degradation of highly chlorinated solvents, but does not indicate whether the degradation occurs. Therefore, the dechlorination products are the most obvious indicators of the actual occurrence of anaerobic dechlorination at the site. It is specifically important to monitor the occurrence of e.g. ethene and ethane as indicators of complete dechlorination.

The sequential formation of successively more dechlorinated daughter products means that the daughter products can be formed in relatively high concentrations, and it implies a potential possibility for quantification of the *in situ* degradation.

The existence of commercial and industrial sources for the dechlorination products should be kept in mind, including their possible occurrence as impurities in the parent compounds. Special attention should be paid to the potential accumulation of daughter products, since they can be dead-end products in some cases.

4.4 Phenoxy acids

4.4.1 Degradation of phenoxy acids

Phenoxy acids constitute a group of herbicides, which in general are degradable under aerobic conditions. 2,4-D is by far the most studied compound but also MCPP, MCPA, dichlorprop, and 2,4,5-T have been investigated. Also, aerobic studies are much more numerous than anaerobic studies. The aerobic degradability of these phenoxy acids have been shown in several studies [24,46,60,65,88,110,146,148], although the investigations by Pedersen (2000) [110] showed recalcitrance of MCPP and dichlorprop in some of the aerobic incubations. In anaerobic aquifers recalcitrance of phenoxy acids seems to be most likely [2,7,60,81,110,121]. However, some investigations have reported degradation of 2,4,5-T under methanogenic conditions [47], and a few observations of anaerobic degradation of MCPP, dichlorprop and 2,4-D also exist [61,161].

The degradation pathways reported in the literature for 2,4-D, dichlorprop, MCPA, MCPP, and related chlorophenols were reviewed by Reitzel et al. (2004) [**IV**]. The key metabolite in the degradation of all these phenoxy acids is the corresponding chlorophenol resulting from cleavage of the ether bond (2,4-dichlorophenol from 2,4-D and dichlorprop, and 4-chloro-o-cresol from MCPA and MCPP [22,35,46,48,61,62,88,89,104,109,112,129,131-133,141,161]). Under aerobic conditions, the next step is hydroxylation to form for example 3,5-dichlorocatechol or 3-methyl-5-chlorocatechol, and ring opening [46,88,112,127,141]. Under anaerobic conditions the degradation was found to involve dechlorination [22]. For 2,4-D and MCPA, other pathways than those going through the corresponding chlorophenol have been shown, such as the dechlorination of 2,4-D to 4-CPA [22], or ring hydroxylation before ether bond cleavage [88].

4.4.2 Identification of indicator metabolites

Based on the degradation pathways for the phenoxy acids (2,4-D, dichlorprop, MCPA, MCPP), candidates for use as *in situ* indicators of biodegradation could be: 2,4-dichlorophenol and 4-chloro-o-cresol formed by ether bond cleavage, 3,5-dichlorocatechol and 5-chloro-3-methylcatechol from the subsequent hydroxylation, and dechlorination products (4-CPA, 4-chlorophenol, and 2-chlorophenol were shown to be dechlorination products of 2,4-D and 2,4-dichlororphenol, but dechlorination of e.g. dichlorprop might also be possible).

All the candidates have retained most of the parent compound structure, and therefore they can only be formed by degradation of a rather limited number of compounds. Thus, they conform relatively well to the criteria of an unequivocal biochemical relationship to the parent compound.

Several of the suggested metabolites have commercial or industrial sources. 2,4-dichlorophenol is reported to be used in organic synthesis [154] and to be an intermediate in the manufacturing of different pesticides [101], whereas 4-chloro-o-cresol is reported to be an impurity of technical grade MCPA [154]. A further look into the herbicide production history revealed that these chlorophenols, as well as various other chlorophenols and phenoxy acids of no herbicidal use, could be present in substantial amounts as impurities in the phenoxy acid herbicides [**IV**]. The possible dechlorination products are among the impurities, but apart from this, 4-CPA is a plant growth regulator, and 2-chlorophenol is used as a disinfectant, a solvent for polyester fibers, and for organic synthesis [145].

The chlorocatechols have no commercial or industrial uses, but their possible formation during e.g. pulp bleaching constitutes an anthropogenic source, resulting in the presence of different chlorocatechols in paper mill effluents [116]. But for the assessment of biodegradation in groundwater contaminant plumes, this source is most likely to be of minor relevance.

Phenoxy acid herbicides and chlorophenols are frequently detected in groundwater, which is why the suggested metabolites with phenoxy acid or phenol structure can be expected to be biologically and chemically stable. Chlorocatechols might be relatively unstable, but very little is known about their distribution and fate in soil [126] and groundwater.

In the majority of laboratory experiments concerning phenoxy acid degradation, the suggested indicator metabolites are degraded, though accumulation of chlorophenols were observed in a few cases [22,48] under anaerobic conditions. However, the same chlorophenols were in other studies found to be degradable under anaerobic conditions [8,55,85,138,139].

Overall, the chlorocatechols might be useful *in situ* indicators of aerobic phenoxy acid and chlorophenol biodegradation. However, their stability in groundwater needs to be investigated in order to assess whether they are likely to occur in measurable concentrations. The other suggested candidates (chlorophenols and dechlorinated phenoxy acids) fail as specific *in situ* indicators.

4.4.3 Impurity/parent compound ratios

The synthesis of phenoxy acid herbicides consists basically of two steps as shown for dichlorprop in Figure 4.5: (1) Chlorination of phenol to 2,4-dichlorophenol (and other chlorophenols), or o-cresol to 4-chloro-o-cresol (and other chlorocresols), and (2) Reaction with chloroalkanoic acid to give the corresponding phenoxyalkanoic acid (of each present chlorophenol). In the early years of production, chlorination was performed without a catalyst, and the products of each reaction step as well as the final precipitate were not purified. Based on this, Reitzel et al. (2004) [**IV**] estimated that the fraction of the desired phenoxy acid in the product could theoretically be as low as 50% for MCPA and MCPP and 62% for 2,4-D and dichlorprop (Figure 4.5). In

contrast to this, the purity of more recently produced phenoxy acids will be 95-96% with 1-2% chlorophenols.

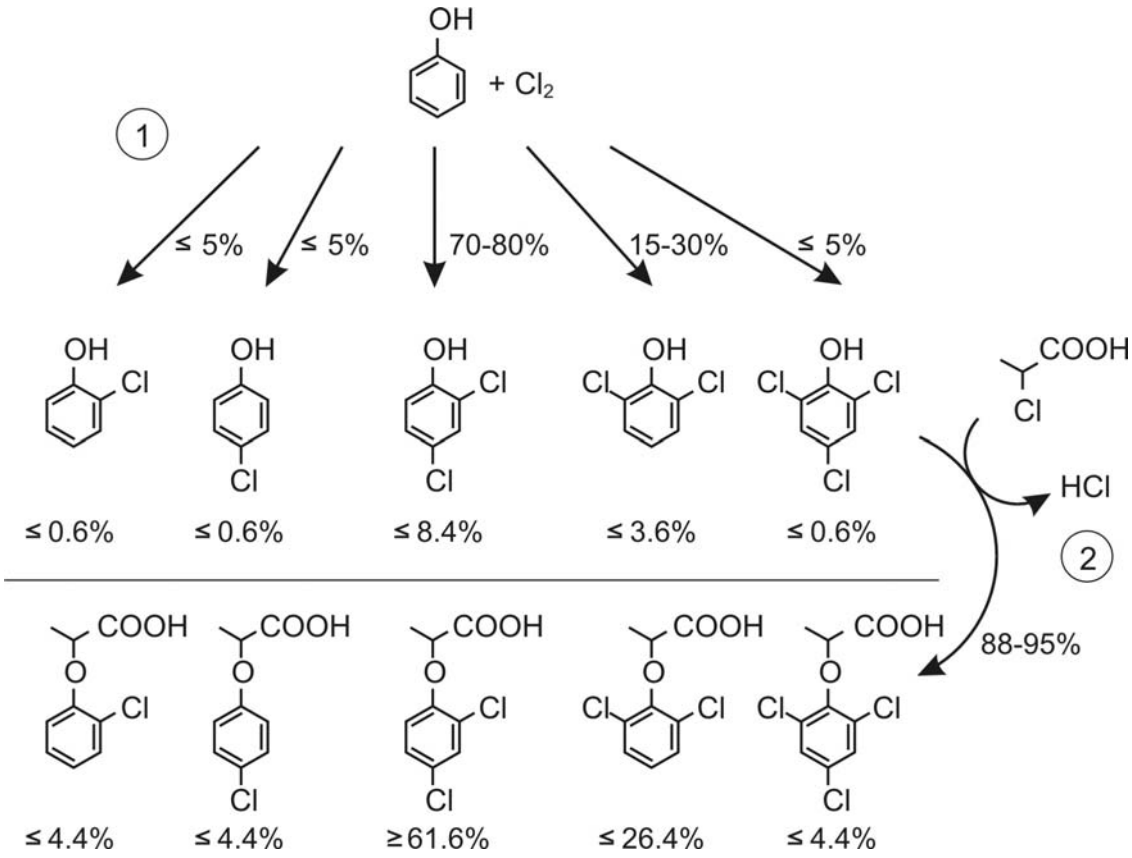

Figure 4.5 The synthesis of dichlorprop anno 1950. The initial chlorination of phenol (1) leads to (from left to right): 2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, and 2,4,6-trichlorophenol in the relative amounts indicated next to the arrows. The subsequent reaction with 2-chloropropanoic acid (2) converts 88-95% of the chlorophenols to (from left to right): 2-chlorophenoxypropanoic acid (2-CPP), 4-chlorophenoxypropanoic acid (4-CPP), 2,4-dichlorophenoxypropanoic acid (**dichlorprop**), 2,6-dichlorophenoxypropanoic acid (2,6-DCPP), and 2,4,6-trichlorophenoxypropanoic acid (2,4,6-TCPP). The percentages below the compounds correspond to the worst-case content of each compound (max. impurity content, min. dichlorprop content) as regarded separately.

A phenoxy acid contaminated site could be polluted with herbicides originating from different periods, and the composition of the source (spilled or deposited herbicides) is often not known. To know the maximal fraction of impurities that could

be present in the initial source, the worst-case ratios of the impurities relative to the parent herbicides can be calculated [**IV**]:

$$\text{worst}-\text{case ratio} = \frac{\text{worst}-\text{case (highest) impurity content}}{\text{worst}-\text{case (lowest) parent herbicide content}} \qquad (4.1)$$

A contamination plume from a continuous source would consist of an area with full breakthrough of both phenoxy acids and chlorophenols and therefore the same impurity/parent herbicide ratios as in the source if we neglect degradation and assume that dilution and sorption are the only attenuation processes. Further downgradient there may be an area with breakthrough of phenoxy acids, but not of the more retarded chlorophenols, therefore the relative concentrations of chlorophenols would be smaller than at the source. Hence, the impurity/parent herbicide ratio is expected to be constant, or in the case of a chlorophenol/parent herbicide ratio declining, with increased distance from the source. This in turn means, that only degradation can cause the impurity/parent herbicide ratio to increase. If the impurity is actually produced as a metabolite of degradation at a field site, it could lead to an increase not only in the ratio, but also in the absolute concentration of that impurity.

These considerations led to the suggestion of three ways to use impurities as *in situ* indicators:

(1) If the observed ratio in a contaminant plume exceeds the worst-case ratio, degradation of the parent herbicide is indicated, independent of the flowpath.

(2) Changes in an impurity/parent herbicide ratio along a flowline could indicate degradation. In the case of chlorophenol impurities the change needs to be an increase, since a decreasing ratio could be due to the different mobility of chlorophenols and phenoxy acids.

(3) An increase in the absolute concentration of an impurity along a flowline indicates that it is being produced as a metabolite, and therewith that degradation of the parent herbicide has occurred.

The exceeding of worst-case ratios at the Sjoelund and Bornholm field sites was substantial even in the plume cores [**IV**], and evidently indicated the *in situ* biodegradation of phenoxy acid herbicides. On the other hand, a clear-cut trend in the observed impurity/parent herbicide ratios was not obvious, but at both sites the most significant changes seemed to occur at the plume edges. For the Sjoelund site this added to a previous demonstration of phenoxy acid mass removal, which was shown to be primarily occurring at the leading edge (between 50 and 100m downgradient), while insignificant in the anaerobic plume core.

Based on the analysis of leachate and leachate-contaminated groundwater from ten Danish landfills [11], two cases of the 4-CPP/dichlorprop ratios above the worst-case ratio, one case of the 2,6-DCPP/dichlorprop ratio, and eight cases of the 4-chloro-*o*-cresol/(MCPP+MCPA) ratios above the worst-case ratios could be identified (Table 4.2), thus suggesting that dichlorprop and MCPP and/or MCPA is being biodegraded

in the leachates. At the Vejen landfill, MCPP was shown to be recalcitrant in the investigated (anaerobic) part of the plume and consistently, the 4-chloro-o-cresol/MCPP ratio never exceeded the worst-case ratio. On the contrary, this impurity/parent herbicide ratio was decreasing with distance from the source, indicating the attenuation of 4-chloro-*o*-cresol, which might result from either sorption or degradation.

Table 4.2 Concentrations (µg/L) of phenoxy acid herbicides and related impurities found in leachate samples from ten Danish landfills (data extracted from Baun et al. (2004) [11]), and calculated impurity/parent herbicide ratios. Bold figures indicate ratios above the worst-case ratio [**IV**].

	Landfill No.										
Compound	1	2	3	4	5	6	7	8	9	10	LOD
Dichlorprop	0.87	0.93	2.8	5.2	1.3	0.66	0.27	0.38	b.d.	b.d.	0.15
4-CPP	×	15	19	×	×	×	×	×	×	×	0.10
2,6-DCPP	×	1.3	0.74	×	×	×	×	×	×	×	0.10
MCPA	b.d.	0.22	0.46	9.1	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	0.20
MCPP	23	6.7	28	15	5.2	16	b.d.	150	13	0.84	0.25
4-chloro-*o/m*-cresol*	4.6	8.7	10.2	5.6	3.3	6.7	3.1	3.3	1.2	3.6	0.01
Impurity/parent herbicide ratio											Worst-case ratio
4-CPP/ dichlorprop ratio	n.a.	**16**	**6.8**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.07
2,6-DCPP/ dichlorprop ratio	n.a.	**1.4**	0.26	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.43
4-chloro-*o/m*-cresol/ (MCPA+MCPP) ratio	**0.20**	**1.3**	**0.36**	**0.23**	**0.63**	**0.42**	**∞**	0.02	0.09	**4.3**	0.14

* 4-chloro-*o*-cresol and 4-chloro-*m*-cresol were co-eluting in the GC/MS method [**I**], but most likely the peak corresponded exclusively to 4-chloro-*o*-cresol, since 4-chloro-*m*-cresol is a pesticide, which is not on the list of the 200 most sold pesticides in Denmark (1956-1993) [100,101].

×: not included in analyses; b.d.: below detection limit (LOD); n.a.: not available.

4.4.4 Discussion

The evaluation of phenoxy acid metabolites revealed that the initial degradation products (corresponding chlorophenols from ether bond cleavage or less chlorinated phenoxy acids resulting from dechlorination under anaerobic conditions) could not be used as specific *in situ* indicators of biodegradation, because they are originally present as impurities in the phenoxy acid herbicides. In other words, the presence of these compounds is not necessarily due to degradation, and in general they should not

be regarded as metabolites when they occur in the environment unless their *in situ* formation can be directly proved.

3,5-dichlorocatechol and 5-chloro-3-methylcatechol might be better candidates for the use as specific indicator metabolites. Their possible occurrence will indicate *in situ* biodegradation of the corresponding chlorophenols, which in turn might be either metabolites of phenoxy acids or original contaminants due to their presence as impurities in the herbicides. However, little is known about the stability and occurrence of these chlorocatechols in groundwater, which is why it is recommended that this issue being further investigated.

Whereas some of the suggested compounds failed as specific metabolites, the impurity/parent herbicide ratios to this end were found to be very useful and easily applied *in situ* indicators of phenoxy acid degradation. Comparisons of impurity/parent herbicide ratios with worst-case ratios can provide evidence of degradation even in the absence of a well-defined flow pattern.

4.5 Summary and perspectives

For a number of biodegradable organic compounds it will be possible to identify or suggest intermediate degradation products. The suitability of these degradation products as *in situ* indicators of biodegradation can be evaluated according to the four characteristics of an ideal indicator (Table 4.1).

The degradation of an organic compound usually occurs in a series of steps, causing a successive disappearance of the structural characteristics of the original compound. Therefore, the early intermediates are more likely to show a unique relationship with the parent compound, while later intermediates can be related to continuously larger groups of compounds, and eventually CO_2 is the final degradation product of any oxidative degradation pathway leading to complete mineralization.

Many potential metabolites will probably be very transient and therefore fail with respect to the criterion for biological and chemical stability. They might not even be released to the extracellular medium. In any case they will not be likely to exceed the detection limits of available analytical methods.

But, having identified an assumed metabolite, which can be measured in the environment, the other decisive factor is the possible existence of commercial and industrial sources and, not the least, the potential occurrence of the assumed metabolite as impurity in the parent compound. As illustrated by the phenoxy acid example [**IV**] (section 4.4) the importance of the latter should not be underestimated. On the other hand, having identified an impurity of a parent compound implies the possible use of the impurity/parent compound ratio as an *in situ* indicator, although the effect of differences in physical/chemical properties should be considered carefully.

At some sites it might be possible to measure the actual impurity/parent compound ratio in the source, which will make it easier to identify downgradient changes in the ratio. In the case of phenoxy acids, it was feasible to estimate a worst-case ratio, the exceeding of which was indicative of degradation, since the impurities were either equal or less mobile than the parent compounds. Therefore, only degradation would cause their relative increase with distance from the source, while sorption would not affect or decrease the ratio. The estimation of a worst-case ratio might be a useful approach for other pairs of impurity and parent compounds as well, but there will also be a number of cases, where the impurity or metabolite is the less retarded compound (e.g. DCE and VC compared to PCE and TCE, 3,5-dichlorophenol compared to pentachlorophenol, or benzoates compared to monoaromatic hydrocarbons), causing the impurity/parent compound ratio to increase with distance from the source even in the absence of degradation. Yet, some compounds such as the chlorinated aliphatic hydrocarbons form metabolites in so large quantities when degradation is occurring, that the potential existence of the metabolites as impurities in the parent compounds is obviously irrelevant at some sites.

The last characteristic of an ideal indicator of biodegradation is being an intermediate in the degradation pathway rather than a dead-end product. However, this is not an inherent property of the metabolite, since some metabolites can be formed either as intermediates or as dead-end products depending on the environmental conditions and/or the microbial species involved. Field sites are often characterized by spatial variations in redox conditions, and a specific microbial community. Thus, in order to rely on *in situ* biodegradation as an efficient attenuation process, at each specific field site it should be rendered probable that the original contaminants as well as the produced metabolites are being further degraded and eventually mineralized within a reasonable distance from the source of contamination.

5 Naturally occurring isotopes

5.1 Isotope ratios

5.1.1 Isotopes in the environment

Many elements consist of two or more naturally occurring isotopes. Some of the common elements existing as more than one isotope are hydrogen, carbon, nitrogen, oxygen, sulphur and chlorine. The average natural abundances of the different isotopes are shown in Table 5.1.

Behind these average abundances lies a great variation between different environments and different species. Furthermore, different processes can change isotopic compositions. In this chapter the focus will primarily be on the stable carbon isotopes, ^{12}C and ^{13}C.

Table 5.1 Natural isotopic abundances (%) of some common elements (Extracted from McLafferty and Turecek (1993)[96]).

	A		A+1		A+2	
Hydrogen	^{1}H:	99.985	^{2}H:	0.015	$^{3}H^{*}$:	10^{-14} to 10^{-16}
Carbon	^{12}C:	98.90	^{13}C:	1.10	$^{14}C^{*}$:	~ 10^{-10}
Nitrogen	^{14}N:	99.63	^{15}N:	0.37		
Oxygen	^{16}O:	99.76	^{17}O:	0.04	^{18}O:	0.20
Sulphur	^{32}S:	95.02	^{33}S:	0.75	^{34}S:	4.21
Chlorine	^{35}Cl:	75.77			^{37}Cl:	24.23

5.1.2 Assessment of biodegradation using isotope ratios

The characteristic isotopic composition of different sources of carbon and the changes in isotopic compositions of e.g. specific components can be used in different ways in the assessment of the natural attenuation of organic contamination.

One approach is to look at dissolved inorganic carbon (DIC = HCO_3^- + CO_3^{2-} + CO_2, aq) and set up a mass balance. There can be different sources of DIC, e.g. dissolution of atmospheric CO_2 or solid carbonates, degradation of organic compounds etc., and the overall carbon isotopic composition will be defined by the contributions from each of the sources. If the relative contributions are changed it will be reflected in the isotopic composition, and the possible increase in CO_2 derived from the biodegradation of contaminants might be identified.

Another approach is to look at changes in isotopic composition of specific compounds. Such a change is caused by isotopic fractionation and involves that the isotopes distribute unequally between product and reactant in a given process. This

means that the isotopic composition of the residual reactant alters as the reaction proceeds. Isotopic fractionation happens because the mass difference causes the reaction rate of a compound to depend on its isotopic composition [66].

Organic contaminants can be removed from solution by several processes, but the corresponding isotopic fractionations are different. Some studies indicate that a relatively large fractionation is associated with degradation (biodegradation as well as some abiotic degradation processes) compared to physical processes. This implies that measuring the fractionation of specific compounds might be a useful tool in the assessment of natural attenuation.

5.1.3 Instrumentation

$^{13}C/^{12}C$ ratios are measured by an isotope ratio mass spectrometer (IRMS). The species to be investigated (e.g. DIC or DOC) must be converted to CO_2 before introduction to the IRMS. Carbonate samples are usually reacted with 100% phosphoric acid at 25°C, while organic compounds are oxidized to CO_2 at 850 to 1000°C in a stream of oxygen or by an oxidizing agent such as CuO [43,66,66]. From CO_2 gas, ions at masses 44 ($^{12}C^{16}O^{16}O$), 45 ($^{13}C^{16}O^{16}O$, $^{12}C^{16}O^{17}O$) and 46 ($^{12}C^{16}O^{18}O$, $^{13}C^{17}O^{16}O$) are produced [18]. Samples are measured relative to a standard gas with known isotopic composition, and thereby it is possible to calculate the ^{13}C content of the sample.

Samples of contaminated groundwater normally contain several different organic compounds, which must be separated in order to measure the $^{13}C/^{12}C$ ratios of the specific compounds. By classical separation techniques this would be quite difficult and time consuming, large sample amounts would be required and the resulting compound specificity would be limited. However, by the coupling of the IRMS to a gas chromatograph (GC) it has become relatively easy to measure the $^{13}C/^{12}C$ ratios of specific organic compounds [18,56,56,84,84]. The GC and the IRMS are connected through a combustion furnace, why the technique is called GC-combustion-IRMS (GC/C/IRMS). Compounds eluting from the GC column are completely converted to CO_2 and water on their passage through the furnace. The combustion is performed at 900°C and catalyzed by CuO granules and the produced water is removed in a cryogenic trap.

The technique is restricted to compounds that are easily separated by gas chromatography, i.e. relatively volatile and apolar compounds. A broader applicability of specific compound isotope analysis is therefore dependent on the development of a similar on-line coupling of HPLC and IRMS. Some successful attempts have been done in recent years [80], but the technique is still in its infancy.

5.1.4 Notation of isotopic ratios and changes in isotopic ratios

The precision of a measurement of absolute isotope abundances is substantially poorer than that of a relative difference in isotope abundances between two samples. Therefore, $^{13}C/^{12}C$ ratios (R) are normally reported relative to a standard (st) using the delta notation ($\delta^{13}C$) [32]. The $\delta^{13}C$ value has units of per mille and is defined as:

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{st}}{(^{13}C/^{12}C)_{st}} \cdot 1000 = (R/R_{st} - 1) \cdot 1000 \qquad (5.1)$$

The standard for reporting carbon isotope data is PDB (Peedee belemnite), which is the carbonate skeleton of a belemnite from the Cretaceous Peedee formation in South Carolina [52,66,66]. The absolute isotope ratio of PDB is $^{13}C/^{12}C \cdot 10^6 = 11237.2 \pm 2.9$ [66]. The availability of PDB is limited, why the use of a secondary standard (calibrated to PDB) such as NBS-19 (TS limestone), NBS-20 (Solenhofen limestone, $\delta^{13}C_{NBS\text{-}20/PDB}$ = -1.06‰) and NBS-21 (spectrographic carbon, $\delta^{13}C_{NBS\text{-}21/PDB}$ = -27.79‰ or –28.10‰) has become common [45,45,52]. To convert $\delta^{13}C$ values from one standard to another, the following equation may be used:

$$\delta^{13}C_{X-A} = \left[\left(\frac{\delta^{13}C_{B-A}}{1000} + 1\right)\left(\frac{\delta^{13}C_{X-B}}{1000} + 1\right)\right] \cdot 1000 \qquad (5.2)$$

where X represents the sample, A and B different standards.

Changes in isotope ratios associated with a reaction are called isotopic fractionation and are expressed by the isotopic fractionation factor, α. Isotopic fractionation of a process can be described in terms of fractional distillation as is expressed by the Rayleigh equation [3,3,64,64,66,86,86,150,150]. For a given reaction, the initial $^{13}C/^{12}C$ ratio of the reactant is $R_{Reac,0}$, and at a given time the $^{13}C/^{12}C$ ratios of reactant and product are R_{Reac} and R_{Prod}, respectively. The Rayleigh equation, describing the isotopic composition of the residual reactant relative to the initial isotopic composition, is then:

$$\frac{R_{Reac}}{R_{Reac,0}} = f^{\alpha-1} = \frac{\delta^{13}C + 1000}{(\delta^{13}C)_0 + 1000} \qquad (5.3)$$

where f is the fraction of residual reactant, and α is given by R_{Prod}/R_{Reac}.

For an irreversible reaction such as biodegradation, the isotope fractionation always goes in the same direction; the product being isotopically lighter than the reactant, and the residual reactant becoming enriched in the heavy isotope. Thus, α is always less

than unity. However, it should be noted, that the inverse definition of α is used in some studies.

Another term used to describe isotopic fractionation is the enrichment factor, ε, which expresses the fractionation in units of per mille [52,52,86]. The enrichment factor is easier to comprehend, because it approximately equals the difference, Δ, between the $\delta^{13}C$ values of product and reactant:

$$\varepsilon = 1000 \cdot (\alpha - 1) \approx 1000 \cdot \ln \alpha \approx \delta_A - \delta_B = \Delta_{AB} \tag{5.4}$$

5.1.5 Some typical $\delta^{13}C$ values for different carbon sources

Knowledge of the $\delta^{13}C$ values for different carbon sources can be used for several applications such as the identification of the source of a specific carbon-containing species, e.g. the origin of a contaminant, or for setting up a carbon isotope mass balance for a system. Some typical $\delta^{13}C$ values with relevance for terrestrial environments are summarized in Table 5.2.

5.2 Changes in the carbon isotope ratio of DIC/CO_2: A mass balance approach

5.2.1 Sources of DIC

The stable carbon isotope ratios of DIC/CO_2 might be used in the verification of the mineralization of hydrocarbon contaminations. The DIC in groundwater or the CO_2 in soil gas can originate from atmospheric CO_2 and carbonate minerals or from the degradation of organic carbon from natural or anthropogenic sources. As indicated in Table 5.2, different sources of DIC/CO_2 have more or less different isotope ratios. Assuming the same isotope ratio for DIC as degradation product as for the organic precursor, the overall carbon isotope ratio for groundwater DIC will be the weighed average of the different isotope ratios associated with the different sources of DIC. Thus, the $\delta^{13}C$ value of DIC provides information about the sources of DIC, which implies the possibility to quantify the amount originating from the degradation of contaminants at sites, where the $\delta^{13}C$ value of the contaminant differs substantially from the $\delta^{13}C$ values associated with other sources of DIC.

The assumption about the DIC produced from the degradation of organic matter having the same isotope ratio as the degraded compound is not always true. Sometimes the degradation can be associated with isotopic fractionation (further discussed in section 5.3), resulting in the degradation product (e.g. CO_2) having a

Table 5.2 Reported $\delta^{13}C$ values of different materials.

Source	$\delta^{13}C$ value
Atmospheric CO_2	-7.4‰ to -12‰ [1] -7 ± 1‰ [52] -7‰ [38,38,43,43,45] -7‰ to -10‰ [4] -6.4‰ to -7.0‰ [108]
Carbonate mineral	+0.7‰ [72] -1.1‰ [20] ~ 0‰ [4] +0.74‰ [9]
CO_2 produced from dissolution of carbonate mineral	-10‰ to -15‰ (DIC) [52] -10‰ (CO_2, g) [4]
C3 plants	-24‰ to -34‰ [43] -27‰ [52] -25 ± 5‰ [1] ~ -25‰ [4,38] [38]
CO_2 produced from root respiration of C3 plants	-19‰ to -22‰ [52] ~ -25‰ [4]
C4 plants	-6‰ to -19‰ [43] -13‰ [52] -5.6‰ to -18.6‰ [1] -12 ± 5‰ [4] -20‰ [38]
CO_2 produced from root respiration of C4 plants	~ -10‰ [52] -12 ± 5‰ [4]
methane	-110‰ to –13‰ [152,155] [155]
CO_2 produced under methanogenic conditions	-12 ± 4.83‰ [155] +10.3‰ to +20‰ [152] +38‰ [52]
Fossil fuel hydrocarbons	-21‰ to -32‰ (oil) [45] -20‰ to -34‰ (petroleum) [1] -18‰ to -34‰ (petroleum) [43] -26‰ (petroleum) [38] -25‰ (coal) [38,38,43] -27 ± 2.3‰ (gasoline) [1]
Benzene	-24.77‰ to -29.40‰ [59]
Toluene	-26.40‰ to -29.03‰ [59]
Ethylbenzene	-26.17‰ to -27.75‰ [59]
o-xylene	-27.26‰ to -28.47‰ [59]
m-xylene	-26.09‰ to -26.30‰ [59]
p-xylene	-26.20‰ to -26.56‰ [59]
PCE	-23.19‰ to -37.20‰ [153]
TCE	-27.80‰ to -31.90‰ [153]
1,1,1-Trichloroethane	-25.80‰ to -29.42‰ [153]

lower ^{13}C content than the residual organic compound. Neglecting the possible isotope fractionation may result in an underestimation of the amount of DIC originating from degradation. Methanogenic degradation is a special case, because it results in the formation of CO_2, which is enriched in ^{13}C (see Table 5.2) compared to the organic compound being degraded, while the produced methane is depleted. This in turn means that CO_2 produced from methane oxidation can be very depleted in ^{13}C, because the methane substrate is often depleted itself, and because methane oxidation can be associated with pronounced carbon isotope fractionation (enrichment factors of e.g. 13.0-25.2‰ [29]/5.0-29.6‰ [10]. Thus, in a carbon isotope mass balance the DIC from methanogenesis and methane oxidation should be regarded as separate sources of DIC.

5.2.2 DIC stable isotope mass balances

As long as only two sources of groundwater DIC or soil gas CO_2 can be identified as significant contributors, the mass balance approach is quite straightforward, and the amount of DIC/CO_2 derived from contaminant degradation can be directly calculated [4,74,152].

At a field site in Studen (Switzerland), where the aquifer was contaminated from a spill of heating oil [20], a graphical method was used to subtract the background (bg) from the measured DIC (meas) and isolate the $\delta^{13}C$ of the DIC increase (inc). From the mass balance:

$$\delta^{13}C_{meas} \cdot DIC_{meas} = \delta^{13}C_{bg} \cdot DIC_{bg} + \delta^{13}C_{inc} \cdot DIC_{inc} \qquad (5.5)$$

the $\delta^{13}C_{inc}$ can be found as the slope of a linear fit to a plot of $\delta^{13}C_{meas} \cdot DIC_{meas}$ vs. DIC_{inc}. However, the approach only holds if $\delta^{13}C_{inc}$ is the same for all the plotted data points, i.e. the relative contributions of different processes to $\delta^{13}C_{inc}$ must be similar. The data from most wells in the field did lie on a straight line, but the data from two wells strongly deviated from this line. These two wells had high concentrations of methane, while the other wells contained little or no methane. It is therefore suggested that such deviations from linearity can be used to identify methanogenic areas. For the other wells, the respective contributions to the increase in DIC were then found to be 88% from hydrocarbon mineralization and 12% from carbonate dissolution.

At a hydrocarbon-contaminated site in Menziken (Switzerland) [72], the expected change in DIC based on EA consumption was under-estimated compared to the observed change in DIC. Since the expected change in alkalinity agreed with the observed change, the disagreement was supposed to be connected to alkalinity-neutral processes, which could be aerobic or methanogenic hydrocarbon degradation or

methane oxidation. These processes will contribute differently to the carbon isotope ratio of the DIC, why the use of isotope ratios is a suitable tool for the differentiation. For one flowpath, the enriched $\delta^{13}C$ value for DIC and the occurrence of methane suggested methanogenesis. For another flowpath a depleted $\delta^{13}C$ value for DIC suggested methane oxidation, while for the other flowpaths the isotope ratios indicated that most of the produced DIC originated from non-methanogenic hydrocarbon degradation.

5.2.3 Combined $\delta^{13}C$ and ^{14}C analysis

In many hydrocarbon-contaminated soils, the $\delta^{13}C$ values of the contaminants and the natural organic matter overlap significantly, and stable isotope analyses can then lead to ambiguous results. Shifts in $\delta^{13}C$ values caused by certain metabolic processes in soil or groundwater, e.g. methanogenesis and methane oxidation, may also complicate the interpretation of results.

One way to solve some of these problems is to combine the stable isotope analyses with measurements of the ^{14}C content of the soil gas CO_2 or the DIC of the groundwater [30,30,31]. Petroleum hydrocarbons as well as the hydrocarbons used for the production of many synthetic compounds (e.g. chlorinated hydrocarbons) are derived from fossil sources and have no ^{14}C, whereas plant material is generally produced in the recent past and will have ^{14}C contents close to atmospheric levels. The ^{14}C content therefore provides an alternative method to distinguish between CO_2 produced from hydrocarbon degradation and CO_2 produced from the degradation of natural organic matter.

^{14}C contents are reported as a fraction of modern, pre-1950, carbon. Values greater than 1 represent samples containing radiocarbon produced during aboveground testing of nuclear weapons. The value for present-day atmospheric CO_2 is 1.14 [30].

In this way *in situ* biodegradation at two sites in California was verified [30,31]. Hydrocarbon mineralization was verified by the low ^{14}C content of the produced CO_2/DIC, and the combined ^{14}C and $\delta^{13}C$ measurements unequivocally indicated zones of methanogenesis and methane oxidation. Furthermore, the low ^{14}C content of DIC even downgradient of the contamination plume [31], indicated that hydrocarbon migration was limited by microbial degradation, while inorganic byproducts (such as DIC) were carried along with groundwater movement. At another site, *in situ* mineralization of chlorinated hydrocarbons was evidenced by a substantially lower ^{14}C content of DIC in the contaminated groundwater compared to the background [76].

5.2.4 Discussion

The different field studies show that carbon isotope data can be very useful in combination with measurements of DIC/CO_2 concentrations, contaminant concentrations and redox parameters. At some field sites the $^{13}C/^{12}C$ ratios can provide quite unambiguous indications of hydrocarbon degradation. At other sites the $^{13}C/^{12}C$ ratios facilitates the interpretation of results by providing an additional mass balance equation, thereby decreasing the number of unknowns (i.e. different sources of DIC/CO_2) relative to the number of equations. If ^{14}C data can be obtained, one more equation is added. The simplicity of such mass balance calculations of course depends on the number of sources significantly contributing to DIC/CO_2 and how easily these sources can be distinguished.

When the mass balance approach is applied, it is often assumed that the isotopic fraction is only significant for methanogenic degradation and methane oxidation. However, processes such as carbonate dissolution and non-methanogenic degradation of organic compounds might also result in isotopic fractionation. The degree of fractionation in the DIC/CO_2 producing processes and how to incorporate the fractionation effects into the isotope mass balance should receive more attention.

5.3 Isotopic fractionation of specific organic compounds

5.3.1 Isotope fractionation

Instead of looking at the isotopic composition of the CO_2 produced, the specific organic compounds being degraded can be followed. This approach implies that the focus will be on the residual reactant of a process, e.g. biodegradation, instead of the product. While the CO_2 production only may indicate the degradation of the bulk contamination, specific compound isotope analysis can provide unique information about specific contaminants, for which the degradation cannot be verified from bulk parameters.

Isotope fractionation is a process related to the mass difference between different isotopes, which causes a slightly lower degradation rate for molecules that are more enriched in the heavy isotope. Therefore, the ^{13}C content of the residual compound will increase, while the reaction product (metabolite) will be ^{13}C depleted relative to the reactant as the reaction proceeds. Isotope fractionation is typically most pronounced for biodegradation processes and some chemical degradation reactions, while physical processes are less likely to be associated with isotope fractionation [59]. This is why it has been suggested that the occurrence of isotope fractionation might be used for the verification of biodegradation [71,86,99].

The fractionation of contaminants including aromatic hydrocarbons (BTEX and PAH), chlorinated aliphatic hydrocarbons, chlorobenzenes and MTBE during biodegradation has been studied in laboratory batch experiments under different redox conditions to obtain isotope enrichment factors, primarily with respect to carbon isotopes, but the more recent studies often include hydrogen isotopes as well.

Isotope ratios have been included in several field investigations, where they proved to be a valuable tool, in combination with measurements of e.g. contaminant concentrations and redox parameters, for distinguishing between contaminant degradation, other attenuation processes, and mixing of contaminant plumes from different sources.

5.3.2 Isotope fractionation in the laboratory

Due to the significant isotope fractionation observed during the degradation of some organic compounds in the laboratory, it has been suggested that a shift in isotope ratio of a specific groundwater contaminant with distance from its source is indicative of *in situ* degradation. Therefore, it is important to investigate in the laboratory, which compounds fractionate during degradation and to what extent, in order to predict field behavior. The laboratory studies can even provide enrichment factors, which can be used to quantify the extent of *in situ* degradation by calculating the residual fraction of contaminant according to eq. 5.3. Thus, isotope ratios are potential qualitative as well as quantitative *in situ* indicators of degradation.

During recent years, isotope enrichment factors have been determined for a relatively large number of compounds (BTEX, PAH, CAH, chlorobenzenes and MTBE) under different redox conditions, especially carbon isotope enrichment factors, but also hydrogen and chlorine isotope enrichment factors. The reviews by Schmidt et al. 2004) [128] and Meckenstock et al. (2004) [97] include detailed compilation lists of enrichment factors for different compounds. An overview is given in Table 5.3.

The actual isotope fractionation associated with a reaction is not entirely understood. The enrichment factors reported (Table 5.3) indicate that the isotope fractionation varies with the specific compound as well as with the redox conditions. But even with the same compound and the same redox conditions (e.g. aerobic toluene degradation), relatively large variation has been reported. It has been suggested that this is due to the fractionation depending on the initial step in the reaction mechanism [42,92,97].

The variation in isotope fractionation implies that the enrichment factor could be different at different field sites, and even in different parts of a field site dominated by different redox conditions. This might introduce a significant uncertainty into the quantification approach.

Table 5.3 Enrichment factors for aerobic and anaerobic degradation of selected groundwater pollutants (extracted from Schmidt et al. (2004) [128] and Meckenstock et al. (2004) [97]).

Compound	Redox conditions	ε ($^{13}C/^{12}C$)	ε ($^{2}H/^{1}H$)
PCE	anaerobic, dehalogenating	-1.8‰ to -5.5‰	
TCE	anaerobic, dehalogenating	-2.5‰ to -13.8‰	
TCE	aerobic	-18.2‰ to –20.7‰	
cis-1,2-DCE	anaerobic, dehalogenating	-14.1‰ to -20.4‰	
VC	anaerobic, dehalogenating	-21.5‰ to -31.1‰	
benzene	aerobic	-1.5‰ to –3.5‰	-11‰ to -12,8‰
benzene	nitrate-red.	-2.0‰ to –2.4‰	-29‰ to -35‰
benzene	sulphate-red.	3.6‰	-79‰
benzene	methanogenic	-1.9	-60‰
toluene	aerobic	0 to -3.3‰	
toluene	nitrate-red.	-1.7‰	
toluene	Fe(III)-red.	-1.8‰	
toluene	sulphate-red.	-0.8 to -2.2	-198‰ to -728‰
toluene	methanogenic	-0.5‰	-12‰ to -65‰
ethylbenzene	nitrate-red.	-2.2‰	
ethylbenzene	sulphate-red.	-3.7‰	
m-xylene	aerobic	-1.7‰	
m-xylene	sulphate-red.	-1.8‰	
p-xylene	aerobic	-2.3‰	
o-xylene	sulphate-red.	-1.1‰ to -3.2‰	
MTBE	aerobic	-1.4‰ to -2.4‰	-29‰ til -66‰
MTBE	anaerob	-4.2‰ to -14.2	

5.3.3 Isotope fractionation at field scale

In general, the carbon isotope enrichment factors for BTEX exhibit only small differences for different anaerobic conditions, while larger differences are observed for hydrogen isotopes. Also, the carbon isotope enrichment factors for chlorinated aliphatics show somewhat larger variations. Thus, for anaerobic BTEX plumes the error associated with the choice of carbon isotope enrichment factor from the literature is probably relatively small, as well as the error associated with the use of one single enrichment factor to describe the entire plume length despite changing redox conditions. Laboratory-derived enrichment factors for toluene and *o*-xylene

were used to quantify the degradation of toluene and *o*-xylene in two different hydrocarbon-contaminated aquifers in Germany [98,118]. The observed isotopic shifts corresponded to ≥99% degradation, and the calculated biodegradation described the observed decreases in concentrations well, indicating that biodegradation was the major attenuation process for these compounds at both sites (Figure 5.1). Isotopic shifts indicative of degradation were observed for other compounds as well, but as no enrichment factors were available for these compounds, their biodegradation could not be quantified.

Meckenstock et al. (2002) [98], Figure 3

Figure 5.1 Stable carbon isotope fractionation during degradation of aromatic hydrocarbons along a monitoring profile of a contaminated site near the city of Ingolstadt (Germany). (■) Toluene concentrations, (▲) $^{13}C/^{12}C$ isotope ratio, (◆) the extend of biodegradation, and (●) theoretical toluene concentrations calculated from eq. 5.3 (From Meckenstock et al. (2002) [98]).

Larger uncertainties should be expected when plumes of chlorinated solvents are treated in the same way, but the obtained estimate of the *in situ* biodegradation can still be very useful. For instance Sherwood-Lollar et al. (2001)[87] calculated the *in situ* biodegradation of TCE using three different enrichment factors (-13.8‰, -7.1‰, -6.6‰) from the literature, with the result ranging from 40.5% to 66.2% biodegradation in one of the downgradient wells. Indeed, the result varies with the choice of enrichment factor, but is still informative.

For the field application of an enrichment factor determined in the laboratory, it is necessary to assume, that the field data, like the microcosm data, follows the Rayleigh model. Some of the reported field experiments indicate that this might be a reasonable assumption. Hunkeler et al. (1999) [71] investigated fractionation during

dechlorination of PCE in both microcosm and field studies, and the fractionation patterns of PCE and its metabolites corresponded well, why it was concluded that microcosm data probably can be used for the interpretation of isotope data obtained in the field. Richnow et al. (2003) [**III**] measured the carbon isotope ratios of different aromatic hydrocarbons in the Vejen landfill plume, and used the field measurements of corresponding concentrations (multiplied by a dilution factor, according to eq. 2.1 [**II**]) and isotope ratios to calculate the enrichment factors. Even though the concentration data indicated a rather complex flow pattern, the plots of $\ln(R_t/R_0)$ against lnf, according to eq. 5.3, were linear with correlation coefficients of 0.8695 and 0.6406 for *m/p*-xylene and ethylbenzene, respectively, which suggests that the Rayleigh model can adequately describe even complex field data. The obtained enrichment factors of -1.5‰ for *m/p*-xylene and -2.1‰ for ethylbenzene are within the range normally found for BTEX compounds.

5.3.4 Source discrimination

At some sites, differences in $\delta^{13}C$ values cannot be correlated with a decrease in concentrations, but seems to be associated with different origins of the hydrocarbons [75,111]. This implies the possibility to distinguish between different sources of hydrocarbon contamination, which is a completely different application of specific compound isotope analysis. However, each application is limited by the other aspect. The variation in $\delta^{13}C$ values due to different origins of hydrocarbon will make it more difficult to discover fractionation effects caused by biodegradation at field sites with different sources of contamination or a source that varies over time. On the other hand, significant fractionation will alter the isotopic "fingerprint" of a specific source of hydrocarbon and cause lower precision in the identification of that specific source.

5.3.5 Discussion

Within its limits of relatively high detection limits (1 mmol carbon or 8-10 mmol hydrogen on column [128]) and the need for complete chromatographic peak resolution, specific compound isotope analysis is a valuable supplement for the documentation of field scale degradation.

The trend is that the degradation of chlorinated aliphatics is associated with larger isotope fractionation than the degradation of hydrocarbons. This is especially true for the lower chlorinated aliphatics, which are normally metabolites from reductive dechlorination of the higher chlorinated aliphatics, and therefore produced as well as further degraded. This implies that the interpretation of isotope data will be more complicated than for parent compounds.

Hydrocarbon contaminations generally consist of numerous different compounds, so measuring the $\delta^{13}C$ values of all compounds will often be too extensive. The measurements might therefore be restricted to critical compounds of special environmental concern.

The isotope fractionation is generally larger for hydrogen than for carbon, but this is partly contradicted by higher detection limits and larger standard deviations (5‰ for hydrogen compared to 0.2‰ for carbon [128]) associated with hydrogen isotope analysis.

As *in situ* indicators, specific compound isotope ratios can give unique qualitative as well as quantitative information about field scale degradation. Improvement of method detection limits (especially for hydrogen isotopes) by instrument optimization or development of appropriate pre-concentration techniques, and the online coupling of HPLC and IRMS will probably lead to an even broader applicability of specific compound isotope analysis.

6 Enantiomers of chiral pollutants

6.1 Chirality

A chiral (Greek *cheir*, hand) compound exists as a pair of enantiomers, which are non-superimposable, but are each others' mirror images. A compound is chiral if it does not contain a plane of symmetry. The most common (though not the only) cause of chirality in organic molecules is the presence of a carbon atom bonded to four different groups. The individual enantiomers have the property of rotating plane-polarized light, while a racemic (50:50) mixture of the two enantiomers will not rotate the plane of polarization. Compounds that exhibit this property are said to be optically active. Some optically active molecules rotate plane-polarized light to the left (counterclockwise) and are said to be **levorotatory** (-). Other molecules rotate the light to the right (clockwise) and are said to be **dextrorotatory** (+). A chiral compound consists of a (-)-enantiomer and a (+)-enantiomer, which in a racemic mixture (±) will counteract each other.

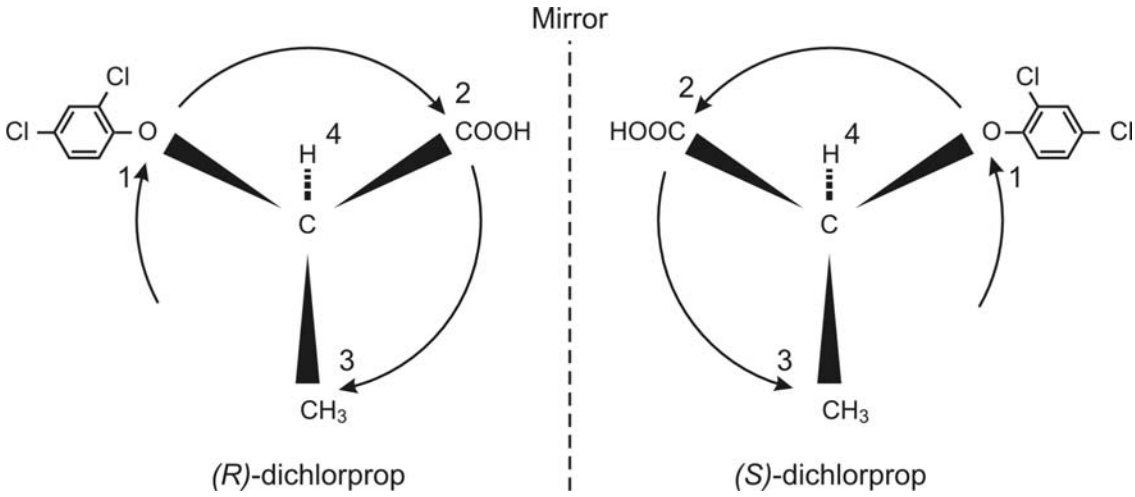

Figure 6.1 The chiral herbicide dichlorprop (2-(2,4-dichlorophenoxy)-propionic acid). Only the *R*-enantiomer is herbicidally active.

The direction of rotation does not say anything about the three-dimensional arrangement (the configuration) of substituents around a chiral center. Instead, this is done by looking at the four atoms directly attached to the chiral center and assigning priorities in order of decreasing atomic number (Figure 6.1). The atom with highest atomic number is ranked first; the atom with lowest atomic number is ranked fourth.

Then the molecule is oriented with the group of lowest priority pointing away from the observer. The three remaining groups are then in a circle. If the substituents of highest rank to second-highest to third-highest rank appears clockwise on the circle, the chiral center has *R* configuration (Latin *rectus*, "right"), and if the substituents appear counterclockwise on the circle, the chiral center has *S* configuration (Latin *sinister*, "left"). There is no simple correlation between the direction of rotation and *R*, *S* configuration.

6.2 Properties of enantiomers

In general, enantiomers have identical physical properties and only differ in the sign of their rotation of plane-polarized light. Other kinds of stereoisomers may have more different physical properties. Enantiomers also behave similarly in chemical reactions, and it should be noted that optically active products (chiral and non-racemic) cannot be produced from optically inactive (achiral or racemic) intermediates or starting materials.

Most biologically important molecules are chiral, and often only one stereoisomer is found in nature. Enzymes are made up of chiral amino acids, and enantiomers can therefore behave very differently in biochemical reactions. This includes the microbial biodegradation of chiral pollutants in the environment, which can be enantioselective, meaning that one enantiomer is degraded at a higher rate than the other. Such enantioselective degradation will cause a change in enantiomeric composition, while abiotic processes such as sorption [95], dilution, and evaporation are non-enantioselective. Therefore, the enantiomeric composition might be used as an *in situ* indicator to demonstrate that biodegradation has occurred in the field.

6.3 Chiral pollutants

Several different potential pollutants are chiral, including pharmaceuticals, surfactants, plasticizers and pesticides. Worldwide it has been estimated that 25% of the pesticides used commercially are chiral compounds [158]. In Denmark, the most sold pesticide (1956-1993) was the chiral herbicide dichlorprop, and out of the 60 most sold pesticides, 7 were chiral and made up 24% of the total weight [100]. The chiral phenoxy acid herbicides dichlorprop and MCPP are among the commonly reported pesticides in groundwater. The metabolism and fate of chiral pollutants were reviewed recently [78,106].

6.4 Degradation of chiral compounds in the laboratory

The chiral herbicides MCPP and dichlorprop are some of the most extensively studied compounds with respect to microcosm experiments dealing with enantiomers. From the degradation experiments with pure cultures of microorganisms it seems that a specific microorganism can be associated with a specific enantioselectivity. Selective degradation of the *(R)*-enantiomers of MCPP and dichlorprop was shown for an *Alcaligenes denitrificans* strain [93,125,140], and for *Rhodoferax* sp. P230 [105]. *Delftia acidovorans* MC1 also preferred the *(R)*-enantiomers, but degraded the *(S)*-enantiomers as well at a lower rate. Selective degradation of the *(S)*-enantiomers of MCPP and dichlorprop was observed for *Ralstonia eutropha* JMP134 and *Burckholderia cepacia* RASC [125], while the preferential degradation of the *(S)*-enantiomers was observed for *Sphingomonas Herbicidovorans* MH, with the *(R)*-enantiomers being degraded at a lower rate [77,162,163]. Thus, all kinds of chiral preferences have been demonstrated in the laboratory regarding the degradation of dichlorprop and MCPP.

The active biomass and the enzymatic systems being expressed can also depend on different environmental conditions, and this might explain why a change in environmental conditions can cause a change in enantioselectivity [83]. Harrison et al. (2003) [61] found that *(S)*-MCPP was degraded faster than *(R)*-MCPP in groundwater under aerobic conditions, while only *(R)*-MCPP was degraded under nitrate-reducing conditions. Zipper et al. (1999) [161] also found the preferential degradation of *(S)*-MCPP as well as *(S)*-dichlorprop when incubated with sewage sludge under aerobic conditions, but in the anaerobic incubations neither the *(S)*- nor the *(R)*-enantiomers degraded. Romero et al. (2001) [120] found the preferential degradation of the *(R)*-enantiomers of MCPP and dichlorprop in sandy soil, but preferential degradation of the *(S)*-enantiomers in clayey soil. Buerge et al. (2003) [25] found that the enantioselectivity for the fungicide metalaxyl in aerobic soil was linearly correlated with pH, the *(R)*-enantiomer being preferred at pH > 5 and the *(S)*-enantiomer being preferred at pH < 4. Their reevaluation of published kinetic data for dichlorprop and MCPP revealed that a similar linear relationship was indicated for these herbicides.

6.5 Evaluation of the enantioselectivity in the field

Enantioselective degradation of chiral compounds seems to be relatively common. During bioremediation of groundwater contamination from point sources, the observation of a change in enantiomeric composition along a flowline might serve as an *in situ* indicator of biodegradation. The enantiomeric composition can be expressed by the enantiomeric ratio, ER:

$$ER = \frac{[R]}{[S]} \tag{6.1}$$

or by the enantiomeric fraction, EF:

$$EF = \frac{[R]}{[R]+[S]} \tag{6.2}$$

where [S] and [R] are the concentrations of the *R*- and *S*-enantiomers, respectively. By using the enantiomeric fraction infinite values are avoided [58]. However, a similar enantioselectivity at different sites should not be expected, since the enantioselectivity depends on the microbial community present at the site as well as the environmental conditions.

Supportive degradation studies might be a possible way to outline the enantioselectivity at a specific site, although the extrapolation from microcosm to field scale is always a challenge. Microcosm experiments in the laboratory often show lag phases or initial phases of slow degradation. The length of the lag phase or the degradation rate in the initial slow phase can vary a lot for different compounds and even for the enantiomers of a chiral compound. Several and diverse reasons for this initial phase have been suggested, such as acclimatisation, growth of specific degraders or expression of certain enzymes.

For contamination plumes in the field, which are undergoing biodegradation, this initial phase will pass within a short time, after which a stationary phase will be reached where attenuation processes including biodegradation complements the flux of contaminants from the continuous source. The growth of microorganisms in traditional inoculation microcosms can be divided into four phases: Lag-phase, exponential phase, stationary phase and death phase. For contamination plumes in the stationary phase, it seems most reasonable to compare only with the stationary phase of the microcosms. This can be done by using the enantioselectivity, ES, as defined by Buerge et al. (2003)[25]:

$$ES = \frac{k_{1,R} - k_{1,S}}{k_{1,R} + k_{1,S}} \tag{6.3}$$

which is a way of expressing the difference in the degradation rate between the two enantiomers, neglecting any initial phase and assuming first-order degradation. Thus, only the linear range of the logarithmic plots, ln[R] or ln[S] versus time is taken into account. The calculated enantioselectivity is a constant in the range of $-1 \leq ES \leq 1$, which might be used to predict the changes in enantiomeric fraction in the field. If $ES > 0$, the *(R)*-enantiomer is preferentially degraded ($k_{1,R} > k_{1,S}$) and EF will decrease, while $ES < 0$ means that the *(S)*-enantiomer is preferentially degraded ($k_{1,R} < k_{1,S}$) and

EF will increase. If other kinetics are assumed (e.g. zero-order, second-order, Monod), the change in EF cannot be predicted from the rate constants alone, but also depends on the relative concentrations of the enantiomers. For instance, assuming zero-order kinetics EF decreases if $k_{0,R}/[R] > k_{0,S}/[S]$, and increases if $k_{0,R}/[R] < k_{0,S}/[S]$.

6.6 Field studies

There are very few field studies including the analysis of the enantiomers of phenoxy acids. In addition to an aerobic field injection experiment revealing non-enantioselective degradation of MCPP and dichlorprop [123], they include the plume from the Helpston landfills in the Lincolnshire Limestone aquifer in the UK (only MCPP) [61,159], a plume from the waste disposal site Kölliken in Switzerland (only MCPP) [164], and the plume from the Sjoelund landfill in Denmark (MCPP, dichlorprop, 4-CPP, 2-CPP) [**VI**].

Immediately downgradient of the Helpston landfills where iron- to nitrate-reducing conditions existed, MCPP was dominated by the *(S)*-enantiomer (EF=0.03-0.11). In accordance with this observation, supportive microcosm experiments [61] showed the selective degradation of *(R)*-MCPP under nitrate-reducing conditions.

In the aerobic part of the aquifer further downgradient, the enantiomeric fraction is somewhat higher (0.30–0.50). The authors explain this by the preferential degradation of *(S)*-MCPP, which they observed in the supportive aerobic microcosms. However, in one aerobic microcosm the degradation rates look quite similar, only the lag phases differ for the two enantiomers. Thus ES=0, and degradation should not cause a change in EF. In the other aerobic microcosm the data were fitted by zero-order kinetics, giving $k_{0,S} > k_{0,R}$. But the MCPP entering the aerobic zone in the field is very depleted in the *(R)*-enantiomer due to the selective degradation of *(R)*-MCPP in the nitrate-reducing zone, and therefore EF should actually decrease, since $k_{0,R}/[R]$ exceeds $k_{0,S}/[S]$ by an order of magnitude. The flow path is probably not very certain, since an area of several km^2 is covered by a very limited number of monitoring wells. A more likely explanation for the increased enantiomeric fraction in the aerobic part of the plume compared to the nitrate-reducing part could be the mixture with racemic MCPP from another source area.

Even further downgradient, an area with sulphate-reducing conditions exists, where an enantiomeric fraction of 0.87 was observed. However, the supportive microcosms under methanogenic/sulphate-reducing conditions could not explain this, since neither *(R)*-MCPP nor *(S)*-MCPP was degraded. Interestingly, an analogy can be made to the groundwater downgradient at the Kölliken waste deposit site, which is also governed by sulphate-reducing to methanogenic conditions. The enantiomeric composition ranged from racemic to EF=0.88. Thus, the field investigations suggest that MCPP, or

at least *(S)*-MCPP, can degrade under sulphate-reducing to methanogenic conditions, though this has never been shown in the laboratory.

At Kölliken the chemical waste was deposited between 1979 and 1985, which was before the introduction of the enantiomeric pure herbicides MCPP-P and dichlorprop-P (containing only the herbicidally active *(R)*-enantiomer) by BASF in 1987 and later by Marks [145,158], why the increased enantiomeric fraction can only be caused by biological processes. The discharge of MCPP into the Helpston landfills occurred during the 1980s, i.e. with the possible inclusion of some years after 1987. However, all measurements within the source area points to a racemic composition.

At the Sjoelund landfill site, which was also closed before the introduction of MCPP-P and dichlorprop-P, the enantiomeric fractions for dichlorprop, 4-CPP and 2-CPP were elevated in most of the plume, while MCPP kept its initial racemic composition everywhere in the plume. Dichlorprop and 2-CPP had EF values of approximately 0.6, but no significant trend was observed with distance from the landfill border. This indicated that enantioselective degradation occurred during the vertical transport from the landfill body to the groundwater, but that either no degradation or non-enantioselective degradation occurred during downgradient migration. On the contrary, EF for 4-CPP was 0.7-0.8 at the border of the landfill, 0.8-0.9 at a distance of 50 m, and 0.15-0.7 at a distance of 100 m, which suggested the continued enantioselective degradation in the groundwater with a shift in enantioselectivity between 50 and 100 m downgradient. As opposed to the Helpston and Kölliken field sites, MCPP at Sjoelund did not show changes in enantiomeric composition.

6.7 Quantification of field scale biodegradation

The Sjoelund landfill site was used to demonstrate how the observed changes in enantiomeric fraction in the field in combination with estimates of the enantioselectivity (ES) obtained from supportive microcosm experiments can be used to quantify biodegradation at the field scale [**VI**]. The residual fraction of a chiral phenoxy acid was calculated as:

$$f = \frac{EF_0}{EF} \cdot \left(\frac{EF_0(1-EF)}{EF(1-EF_0)} \right)^{-\frac{1}{2}\left(\frac{1+ES}{ES}\right)} \tag{6.4}$$

where EF_0 is the initial enantiomeric fraction and EF is the more downgradient enantiomeric fraction for a given flowpath. The residual fraction corresponds to a certain percentage being biodegraded:

$$B = 100 \cdot (1 - f) \tag{6.5}$$

The approach applied to dichlorprop, 4-CPP and 2-CPP, and it was shown that a significant fraction dissipated during the vertical transport from the landfill body through the 15 m thick unsaturated zone to the groundwater. Once in the groundwater, the remaining fraction of phenoxy acids was rather persistent within the anaerobic leachate plume. Only 4-CPP showed changes in enantiomeric fraction under aerobic conditions. Quantification based on these changes showed that 4-CPP was completely degraded under aerobic conditions at the fringes of the plume (Figure 6.2).

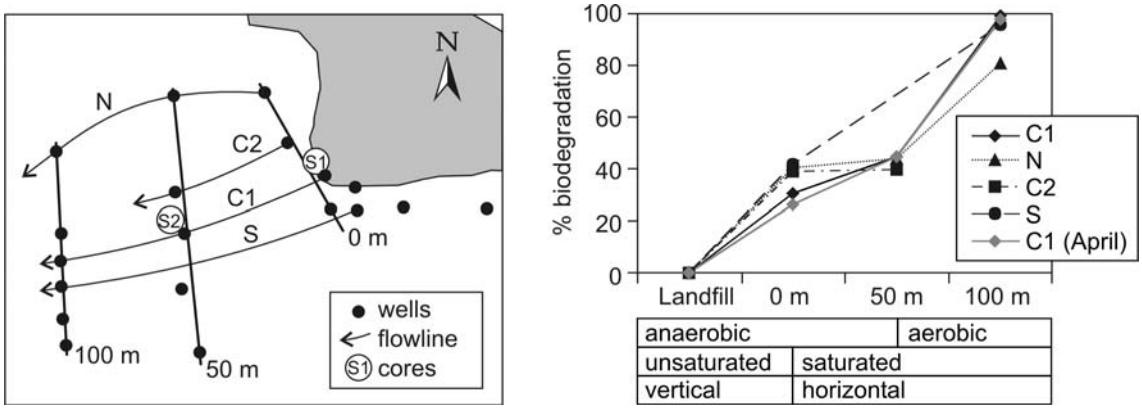

Figure 6.2 Biodegradation of 4-CPP along the flowlines at Sjoelund, quantified according to equations 6.4 and 6.5. All flowlines were sampled in October 2003, and the central flowline C1 was resampled in April 2004. The redox and flow conditions are indicated below the graph.

The question naturally arises of whether it would be possible to use the quantification approach at the plumes from the Helpston landfills [61,159] and the waste disposal site Kölliken in Switzerland [164].

In the iron- to nitrate-reducing area immediately downgradient of the Helpston landfills, the approach is easily applied. The selective degradation of *(R)*-MCPP in nitrate-reducing microcosms would correspond to an enantioselectivity of ES = 1. With this enantioselectivity, the observed change in enantiomeric fraction from 0.5 in the source to 0.03 furthest downgradient in the nitrate-reducing part of the aquifer corresponds to 48.5% biodegradation.

In the aerobic and sulphate-reducing parts of the aquifer the quantification approach becomes problematic, since the flowpath is not very well-defined and observed changes in enantiomeric fraction might be due to mixing of MCPP from different source areas as discussed above. Although the high enantiomeric fraction of 0.87 in the sulphate-reducing part strongly indicates degradation with $0 < ES \leq 1$, the supportive microcosms showed no degradation. Assuming ES = -1 and $EF_0 = 0.5$ the

biodegradation can be calculated to be 42.5%, which is the lowest amount of biodegradation that can be associated with EF = 0.87.

The waste disposal site Kölliken has the same problems, that the flowpath is not very well-defined, and the microcosm experiments show no degradation, although degradation is indicated in the field by increased enantiomeric fractions downgradient of the source area. Again, an enantioselectivity of ES = -1 could be assumed, and the calculated biodegradation would then be 0-43%.

6.8 Discussion

Enantiomeric fractions are relatively easy to measure and apply as a qualitative *in situ* indicator of the biodegradation of chiral compounds. Often a synthetic chiral compound is known to have a racemic composition, why the source or sources of the chiral contaminant can be assigned an enantiomeric fraction of 0.5. In that case, a significant deviation from this initial value in a downgradient sampling point will independently of flow patterns indicate that biodegradation has occurred. Yet, the absence of a shift in enantiomeric fraction is also likely even in the presence of biodegradation, since biodegradation is not necessarily enantioselective.

A trend along a flowline has the potential to provide more detailed information e.g. about the existence of low-degradation and high-degradation areas. In the presence of more than one source, however, an apparent downgradient trend could also be a result of mixing. The interpretation of trends is also complicated by the possibility of a shift in enantioselectivity in response to changes in environmental conditions.

Enantiomeric fractions were also shown to be useful for the quantification of biodegradation. However, a complication factor from a practical perspective is the need for site-specific supporting microcosm experiments.

The use of enantiomeric fractions is limited to chiral compounds. But for a range of chiral compounds including the phenoxypropionic acids, the enantiomeric fractions constitute one of the only useful *in situ* indicators.

7 The combined use of isotope ratios, compound ratios and enantiomeric fractions

7.1 Compound ratios

The use of the ratio between two species leaching from the same source and having similar chemical and physical properties with respect to properties that are relevant to the system is the backbone of most of the *in situ* indicators presented here, i.e. impurity/parent compound ratios, isotope ratios, enantiomeric fractions and other compound ratios. The combined use of different kinds of ratios can provide a more solid documentation of field scale degradation and more detailed knowledge of the processes at a field site.

7.2 Combination of enantiomeric fractions and compound ratios

At the Sjoelund landfill the degradation of 4-CPP, dichlorprop and 2-CPP was quantified from the change in enantiomeric fraction observed in the field, using laboratory-derived enantioselectivities [**VI**]. However, the 4-CPP/dichlorprop ratio, which in its property of impurity/parent compound ratio can be associated with a maximal initial value, i.e. the worst-case ratio [**IV**], indicated that the degradation of dichlorprop was highly underestimated, probably because the applied enantioselectivity was too large. Furthermore, only the degradation of 4-CPP could be calculated for the total plume length, while the degradaton of dichlorprop and 2-CPP could only be calculated for the anaerobic part of the plume, since their degradation turned non-enantioselective under aerobic conditions. As an alternative, the relationship

$$f_P = f_I \cdot \frac{IPR_0}{IPR} \tag{7.1}$$

was used, where f_P and f_I are the residual fractions of I (impurity, i.e. 4-CPP) and P (parent compound, i.e. dichlorprop), respectively, and IPR is the impurity/parent compound ratio. Thus, having estimated the residual fractions of 4-CPP along different approximate flowlines, the residual fraction of dichlorprop and finally 2-CPP could be calculated as well. The degree of biodegradation, $B = 100\% \cdot (1 - f)$ (eq. 6.5), for dichlorprop along the flowlines is shown in Figure 7.1.

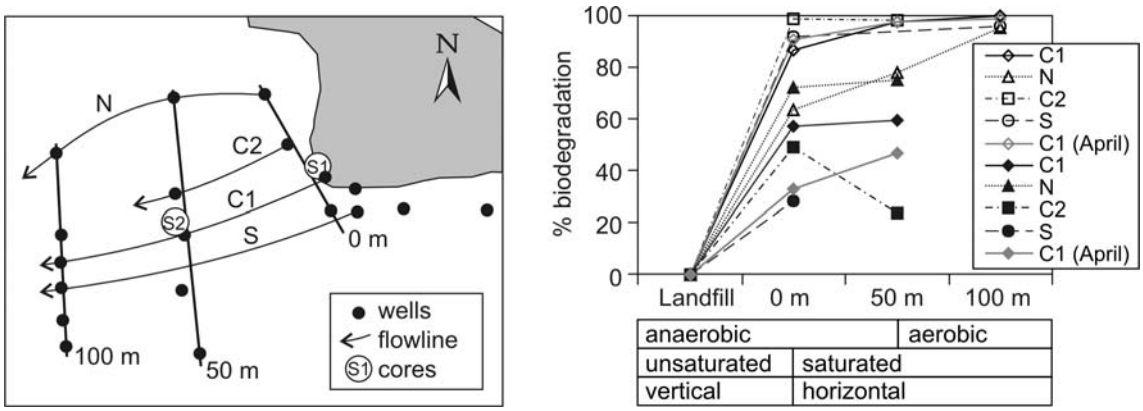

Figure 7.1 Biodegradation of dichlorprop along the flowlines at Sjoelund. Solid symbols correspond to the quantification according to equations 6.4 and 6.5, and open symbols correspond to quantification according to equations 7.1 and 6.5. All flowlines were sampled in October 2003, and the central flowline C1 was resampled in April 2004. The redox and flow conditions are indicated below the graph.

The relationship in eq. 7.1 can easily be generalized to all compound ratios that fulfill the criteria for being used as *in situ* indicators (i.e. same source and similar physical-chemical properties for the two compounds). Thus, defining the fractions of compounds A and B as

$$f_A = \frac{[A]}{[A]_0} \tag{7.2}$$

and

$$f_B = \frac{[B]}{[B]_0} \tag{7.3}$$

the compound ratio is given by

$$\frac{[A]}{[B]} = \frac{f_A}{f_B} \cdot \frac{[A]_0}{[B]_0} \tag{7.4}$$

which rearranges to

$$f_B = f_A \cdot \frac{[A]_0/[B]_0}{[A]/[B]} \tag{7.5}$$

The enantiomeric fraction of MCPP in the plume at Sjoelund did not deviate significantly from the initial value of 0.5, which is why the observed enantiomeric fractions could not be used for quantification of biodegradation. But despite the fact that MCPP and 4-CPP has the same degree of structural similarity as dichlorprop and 4-CPP, the 4-CPP/MCPP ratio cannot be used in the same way as the 4-CPP/dichlorprop ratio. Apparently, 4-CPP and MCPP are leached from two different subsources in the landfill, as two parallel phenoxy acid plumes has been identified within the plume (modeled by Prommer et al., 2004 [113]), one dominated by MCPP and its impurity 2,6-MCPP, and the other dominated by dichlorprop and its impurities 4-CPP, 2-CPP and 2,6-DCPP. Thus, 4-CPP and MCPP do not fulfill the criteria of being associated with the same source. Another problem with this pair of compounds is that the initial ratio is not known at Sjoelund. In the calculations of residual fractions, the landfill body is included as a theoretical sampling point. This is important, since substantial degradation occurs during the vertical transport from the landfill body through the 15m thick unsaturated zone down to the groundwater. Reasonable initial values could be assumed for the enantiomeric fractions (EF = 0.5) and the impurity/parent compound ratios (IPR = worst-case ratio [**IV**]), but the initial 4-CPP/MCPP ratio is unknown.

7.3 Combination of isotope ratios and compound ratios

Whereas enantiomeric fractions can only be defined for chiral compounds, isotope ratios can in principle be measured for any volatile and apolar organic compound. Due to the phenomenon of isotope fractionation, field scale degradation can be quantified from the change in isotope ratio for a compound at the field site, and the enrichment factor determined in the laboratory for that specific compound under the relevant redox conditions (chapter 5.3).

However, some compounds may degrade without significant isotope fractionation, the observed decrease in concentration might be too small for the isotopic shift to be significant, or they co-elute with other compounds in the GC/C/IRMS. For these compounds, it could be considered, whether they can be paired with an isotope-fractionating compound, for which the residual fraction could be calculated from the Rayleigh equation. In that case, eq. 7.5 might be applied.

7.4 Application at the Vejen landfill site

The Vejen landfill is an obvious candidate for testing this concept of combining isotope ratios and compound ratios. Richnow et al. (2003) [**III**] measured the isotope ratios of eight different compounds in eight samples from the first 50 m of the

leachate plume. Four of these became isotopically enriched with distance from the landfill. In order to quantify the degradation, it is necessary to know the enrichment factor. Field-derived enrichment factors for *m/p*-xylene and ethylbenzene was applied in this case, since laboratory-derived enrichment factors were not reported at the time of the study (2000). However, the field-derived enrichment factors and the field concentrations and isotope ratios are inter-dependent, which is why laboratory-derived enrichment factors should be preferred whenever possible. Today, laboratory-derived enrichment factors have been reported for *m*-xylene and ethylbenzene under sulphate-reducing conditions (Table 5.3), and they are used in the following.

Assume that only the biodegradation of one of the two compounds, *m/p*-xylene and ethylbenzene, could be calculated from isotope ratios. Then the biodegradation of the other compound can be calculated using eq. 7.5, if the two compounds fulfill the criteria of being associated with the same source and being equally subjected to physical processes. In that case, a plot of the logarithmic concentrations should be linearly correlated. The plots of ethylbenzene vs *m/p*-xylene show a linear trend (the correlation coefficients are statistically significant at the 95% confidence level), which indicates that they behave relatively similar in the system, although the heterogeneity of the aquifer (and probably of the source as well) provides a scattered picture.

Table 7.1 Calculation of the biodegradation of *m/p*-xylene and ethylbenzene at the Vejen landfill according to equations 5.3 and 7.5, respectively (data taken from Richnow et al. (2003) [**III**]).

Monitoring well	*m/p*-xylene	ethylbenzene	ethylbenzene	*m/p*-xylene
	$\delta^{13}C_{m/p\text{-xylene}}$ (ε = -1.8) (eq. 5.3)	$\delta^{13}C_{m/p\text{-xylene}}$ + compound ratio (eq. 7.5)	$\delta^{13}C_{ethylbenzene}$ (ε = -3.7) (eq. 5.3)	$\delta^{13}C_{ethylbenzene}$ + compound ratio (eq. 7.5)
A	7.7	69.5	30.9	-109.0
B	0.0	0.0	0.0	0.0
C	24.8	63.2	43.7	-15.0
D	83.3	87.8	76.3	67.6
E	76.1	57.0	53.7	74.3
F	56.8	-132.5	-1.4	81.2
G	n.a.	n.a.	51.6	n.a.
H	96.3	92.0	90.5	95.6

n.a.: not available

Table 7.1 shows the biodegradation of *m/p*-xylene and ethylbenzene calculated either directly from the isotope ratio and a laboratory-derived enrichment factor using the Rayleigh equation, or from the fraction of the other compound (as determined from the Rayleigh equation) using eq. 7.5. The well B was chosen to represent the initial composition, since it contained the highest concentrations of specific organic

compounds. Even higher concentrations of ethylbenzene were found in well F along with isotopic depletion, which illustrates the complexity of the flow pattern. This results in a negative biodegradation of ethylbenzene at this well.

Interestingly, the biodegradation as estimated by these two different methods is relatively similar, especially for wells E and H, and indeed leads to the same overall conclusion about the contribution of biodegradation to the plume attenuation. This exemplifies how the combined use of different indicators adds up to give a more evident conclusion.

7.5 Discussion

Generally, in order to convincingly argue that a contaminant plume is attenuated by biodegradation, the biodegradation should be verified by different independent indicators. In this way a better understanding of the subsurface processes is achieved, which is the basis for the safe application of MNA. With respect to the combined use of *in situ* indicators, for instance the combined use of $\delta^{13}C$ and the ^{14}C content of DIC or soil gas CO_2 can add up to provide more conclusive evidence of biodegradation and a better differentiation between processes [30,31], and similarly the combined use of two or more different stable isotope ratios ($\delta^{13}C$, $\delta^{2}H$, $\delta^{37}Cl$, $\delta^{15}N$, $\delta^{34}S$, $\delta^{18}O$) has been suggested [23,42,69,70,92,136].

The simultaneous use of specific metabolites and isotope fractionation has also been suggested [51]. Apart from being two independent indicators of biodegradation, the specific metabolites may be indicative of a certain degradation pathway, which in turn is believed to be associated with a certain isotope enrichment factor [42,97]. In this way, the specific metabolites can serve to improve the quantification based on a shift in the isotope ratio. This illustrates a more integrated way of combining different methods.

The examples given in this chapter, combining a quantitative and a qualitative method in order to obtain quantitative estimates of biodegradation for a larger number of compounds also shows the advantage of integrating different independent *in situ* indicators.

8 Discussion

Before the initiation of any remediation strategy in order to clean up a contaminated site, it is essential to obtain knowledge of the site's geology and hydrology, redox conditions, and distribution and concentrations of contaminants. Depending on the result of this first site investigation, monitored natural attenuation may be suggested as the remediation approach. Thus, the prerequisite for the application of monitored natural attenuation is that attenuation of contaminants is observed in terms of decreasing concentrations, and that degradation of the contaminants are known to be possible under the prevailing redox conditions.

A number of *in situ* indicators, as described in the previous chapters, can be used in the demonstration of biodegradation, in addition to other lines of evidence. The *in situ* indicators are very different in terms of simplicity of application and interpretation, universal validity, analytical limitations, and costs. Neither a single universal *in situ* indicator nor a universal order of application for the list of possible *in situ* indicators can be appointed. Rather, the suitability of the different *in situ* indicators for the demonstration of field scale biodegradation is compound and site specific. It might be feasible, however, to set up some guidelines as to how to choose between the different existing *in situ* indicators.

8.1 In situ indicators for immediate application

Clearly, the more inexpensive indicator tools can be more uncritically and generously applied, while the more expensive approaches naturally will be offered a more careful and thorough consideration before the possible application. Thus, the first *in situ* indicators to be considered could obviously be those that imply no further analyses.

Determination of distribution and concentrations of contaminants was part of the initial site characterization. In some cases it is not feasible to determine the concentrations of all possible contaminants at a site, because the contamination is made up of too many different compounds. Instead, an appropriate bulk parameter such as NVOC or TPH (total petroleum hydrocarbon) accompanied by a reasonable number of target compounds may be determined. Standard commercial analyses often include typically occurring metabolites or impurities. The less chlorinated daughter products of chlorinated solvents as well as the phenoxy acid and chlorophenol impurities are examples of this. A redox characterization was also included in the initial site characterization.

8.1.1 Hydrocarbons

Indication of *in situ* biodegradation might be obtained through the comparison of the concentrations of different hydrocarbons. This has for instance been done by calculating and plotting the compound ratios of pairs of compounds with similar or systematically different physical properties such as K_{ow} or Henry's law constants, double logarithmic plots of pairs of compounds with similar physical properties, or plotting the normalized concentrations for a series of homologues. Indication of biodegradation is by these methods obtained when a systematic trend is observed, which is different from the trend expected if non-destructive attenuation processes dominate.

The occurrence of redox zones correlating with the plume of decreasing hydrocarbon concentrations is a strong indication of bulk hydrocarbon degradation, which can even be quantified from the observed consumption of electron acceptors along a flowline.

8.1.2 Chlorinated solvents

The redox conditions are not indicative of the degradation of chlorinated solvents, but form a basis for the interpretation of other data. A series of dechlorination products, although not necessarily corresponding to the complete dechlorination sequence, is often included in the initial analyses of compound concentrations. Thus, the possible degradation can be assessed based on the sequential appearance of successively more dechlorinated daughter products with distance from the source, in terms of concentrations, molar fractions or dechlorination index.

8.1.3 Phenoxy acids

As with the chlorinated solvents, the redox conditions form a basis for the interpretation of other data, but are not themselves indicative of phenoxy acid degradation. The phenoxy acid impurities and the chlorophenols included in the initial analyses of compound concentrations can be used to calculate impurity/parent compound ratios. Indication of biodegradation is obtained if a systematic change in the ratio is observed along a flowline, or if the worst-case ratio (i.e. the largest possible ratio in the absence of biodegradation) is exceeded in the individual sampling points.

8.2 Evaluation of additional in situ indicators

The use of DIC and alkalinity mass balances, specific degradation products, isotopes or enantiomeric fractions as *in situ* indicators requires additional analyses to be performed. In general, it is recommended to demonstrate biodegradation by different independent methods in order to convincingly argue that *in situ* biodegradation is efficiently attenuating the contaminant plume. Thus, the need for additional *in situ* indicators is obvious. However, the applicability and suitability of the different methods cannot be generalized, but may differ substantially between sites and types of contaminants.

The evaluation of *in situ* indicators performed in this thesis has revealed a number of factors that influence the utility of the different indicators. These factors are summarized in Table 8.1. The listed points for consideration might be useful during a site evaluation, in order to better prioritize and choose between the different *in situ* indicators.

Table 8.1 Factors that may influence the efficacy and suitability of an *in situ* indicator of biodegradation.

Point for consideration	Significance
Bulk contamination or specific compound	Some indicators aim at the bulk organic matter, while others aim at one or more specific compounds. It should be considered whether the bulk degradation or the degradation of some specific compound is the more important one to demonstrate.
Flowpath dependence	Some *in situ* indicators only make sense in terms of a trend along a flowline. Therefore, the applicability depends on how well-defined and well-described the flowline is. The magnitude of the concentration drop along the flowline may also be decisive.
Background	Some *in situ* indicators are to be interpreted by comparison with the adjacent uncontaminated groundwater, which is why the quality of this will influence the applicability of the indicator.
Detection limit	The detection limit of the analytical method can in some cases be decisive. For instance, if a certain concentration change along a flowline is needed, it is crucial that the lower concentrations can be detected.
Availability of the analysis	Some parameters are analyzed by standard procedures and can be measured by any laboratory, while others require highly specialized equipment or expert knowledge.

8.3 Indicators of bulk contaminant degradation

8.3.1 DIC and alkalinity mass balances

The changes in concentrations of DIC and alkalinity are related to bulk contaminant degradation (by oxidation). They can be balanced with the coinciding consumption of electron acceptors, and used to verify or locate sources of error in the mass reduction determined from electron acceptor consumption. A suspicion that the electron acceptor consumption is seriously underestimated could be a reason to apply DIC and alkalinity mass balances. The mass reduction can potentially be calculated as a function of distance from the source, but the method is not truly flowline dependent, since the mass reduction can also be determined from the comparison of contaminated and uncontaminated groundwater at the site. This in turn means that the background concentrations are important since they may camouflage even substantial contaminant degradation. DIC and alkalinity are measured by standard methods and detection limits are not an obstacle.

8.3.2 Carbon isotope composition of DIC

Very much like the DIC and alkalinity concentrations, the isotope ratio of DIC serves to strengthen the demonstration of bulk contaminant degradation and the understanding of the subsurface processes at the site. Also the method is valid with or without a well-defined flowline, but the isotope composition of background DIC needs to be significantly different from the isotope composition of the contaminant. As a supplement to the DIC and alkalinity mass balances, the DIC isotope ratio mass balance provides the opportunity to differentiate between processes that do not differ in terms of DIC and alkalinity production. The application of DIC isotope ratios could be motivated by the distribution of redox parameters being inconclusive, or by a suspicion of an underestimated electron acceptor consumption, maybe confirmed by the DIC and alkalinity mass balances, but not definitively identified with respect to the underlying processes.

Changes in the DIC isotope composition along a flowline may provide information about dilution, since the possible DIC originating from contaminant degradation may be gradually diluted due to mixing with unaffected groundwater. A larger extension of the plume of contaminant-derived DIC compared to the contaminant plume also indicates attenuation of the contaminant by biodegradation. However, the presence of more than two significant sources of DIC can lead to ambiguous results.

The measurement of isotope ratios requires special equipment, which is why the analyses are relatively expensive and only available from a limited number of specialized laboratories.

8.4 Indicators of specific compound degradation: Specific metabolites

8.4.1 Hydrocarbons

In the initial evaluation of *in situ* degradation at a hydrocarbon site the distribution of redox parameters possibly showed that an overall degradation of hydrocarbons in general occurred. Also, the compound ratios for different pairs of specific hydrocarbons might have indicated the preferential degradation of some hydrocarbons. However, the degradation of the least attenuated compounds is not directly verified by these methods. In that case, the possible existence of specific metabolites related to these compounds could be considered, since the occurrence of a specific metabolite provides unequivocal evidence for the degradation of its parent compound.

The succinate derivatives and alkylbenzoates (chapter 4.2) are metabolites of hydrocarbon degradation with no other sources, why there will be no background concentration to correct. This means that the bare presence of these metabolites will indicate biodegradation independently of flowpath or parent compound concentration.

The availability of the analysis could be the limiting factor to the use of these *in situ* indicators of biodegradation. Several of the metabolites are not commercially available as standards, which is why they would need to be synthesized or isolated from microcosms, which either way is time-consuming and costly. Alternatively, the metabolites could be tentatively identified based on their mass spectra, but this requires special expertise and it will not be possible to differentiate between isomers. The metabolites are not necessarily formed in high concentrations in groundwater, but the method detection limits will not be a decisive factor, since the chance for positive identification of metabolites cannot be reasonably predicted.

Lastly, some specific compounds such as benzene do not form specific metabolites upon degradation. Thus, if benzene is the critical compound, the metabolite approach will not provide the desired evidence.

8.4.2 Chlorinated solvents

Some of the daughter products of anaerobic dechlorination were probably included in the initial analysis of contaminant concentrations. If the concentrations of these daughter products indicate the occurrence of anaerobic dechlorination, analysis of the last daughter products may confirm that complete dechlorination to e.g. ethene is occurring and/or that partially dechlorinated daughter products are being accumulated.

The occurrence of ethene will in practice serve as a qualitative indicator of complete dechlorination in relation to a typical contamination with chlorinated

ethenes, and is as such not dependent of the flowpath, parent compound concentration or background. At multiple contaminant sites such as landfills, ethene is probably too unspecific in terms of ideal indicator characteristics. In order to assess the overall efficiency of anaerobic dechlorination at a quantitative or semi-quantitative level, the flow pattern, redox conditions, and the whole sequence of ethenes need to be considered.

The analysis of volatile components such as ethene can be performed by standard laboratories, and, similarly to the hydrocarbon metabolites, the method detection limit will not be the decisive factor.

8.4.3 Phenoxy acids

Unfortunately, the chlorophenols and non-herbicide phenoxy acid, which are often present in relation to phenoxy acid contaminations, fail as specific indicator metabolites because of their possible origin as impurities in the herbicides.

3,5-dichlorocatechol and 5-chloro-3-methylcatechol on the other hand are highly specific for the degradation of 2,4-dichlorophenol and 4-chloro-o-cresol, which are phenoxy acid degradation products and impurities. However, it remains to be investigated whether they are sufficiently stable for the likely detection in contaminated groundwater, which in turn would imply the development of appropriate analytical methods.

Thus, the analysis of specific metabolites associated with phenoxy acids and corresponding chlorophenols is presently not an option.

8.5 Indicators of specific compound degradation: Impurity/parent compound ratios

8.5.1 Hydrocarbons

The term impurity/parent compound ratio cannot be defined for petroleum hydrocarbons.

8.5.2 Chlorinated solvents

The less chlorinated daughter compounds of chlorinated solvents are likely to be minor impurities in the parent compounds, but their presence at contaminated sites are often related to degradation, which is why they are generally treated as metabolites. The use of impurity/parent compound ratios will therefore not really provide new evidence, but the possibility should be kept in mind that the observed concentrations

of assumed metabolites might only correspond to the original content of impurities in the solvents.

8.5.3 Phenoxy acids

A selection of phenoxy acid impurities might have been included if a standard pesticide analysis was chosen for the initial determination of contaminant concentrations, but additional analyses of other impurities might be purchased in order to determine more impurity/parent compound ratios.

The comparison of impurity/parent compound ratios with worst-case ratios in order to detect a possible exceeding has the advantage of being independent of the flowpath, whereas the use of a change in the ratio or an increase in the absolute concentration of a possible metabolite requires a well-defined flowline.

The analysis of a number of phenoxy acids and chlorophenols is available from commercial laboratories.

8.6 Indicators of specific compound degradation: Isotope ratios

8.6.1 Hydrocarbons

In general, the stable isotope ratios may provide evidence for the degradation of potentially any hydrocarbon, both qualitatively and quantitatively. But the different limitations to a successful application should be carefully considered.

The absolute value of the isotope ratio for a specific compound is not very useful. It is the change in isotope ratio along a flowline, which is indicative of degradation. Thus, a relatively well-defined flowline is compulsory, while the existence of multiple sources may lead to ambiguous results. Furthermore, for small decreases in concentration, or if a considerable part of the decrease is not caused by degradation, the isotopic shift due to isotope fractionation may not be significant, because of the analytical uncertainty and especially because of the variations observed at field sites in general. Naturally, a larger decrease in concentration is needed in the case of lower enrichment factors. Therefore, the lists of laboratory-derived enrichment factors for different compounds should be conferred. The degradation of hydrocarbons was in a number of experiments associated with relatively small enrichment factors.

The requirement of a relatively large concentration range along the flowline emphasizes the importance of the method detection limit, which is relative high for GC/C/IRMS measurements compared to the detection limits associated with normal GC/MS or LC/MS analyses. In other words, it is important that the isotope ratio can be determined not only for the plume core samples but also for the more

downgradient samples, which represent the lower concentration of the concentration range.

The GC/C/IRMS instrument needed for the measurement of stable carbon isotope ratios is not standard equipment in most laboratories, which of course will somewhat limit the availability of this kind of analysis.

8.6.2 Chlorinated solvents

Application of the stable isotope ratio approach to contaminations of chlorinated solvents generally has the same limitations as discussed for hydrocarbons. That is, the method is highly dependent on the occurrence of a concentration change of a certain magnitude along a well-defined flowline, and the detection limits and availability of the analysis could be an obstacle as well.

An immediate advantage for chlorinated solvents compared to hydrocarbons is the generally higher carbon isotope enrichment factors associated with their degradation. But while the different hydrocarbons are independent compounds, the chlorinated compounds at a site contaminated with chlorinated solvents will typically include a series of dechlorination products. This series is itself indicative of the degradation of the more chlorinated parent compounds by reductive dechlorination, while it is particularly important to verify the further degradation of the daughter compounds.

Generally, the isotopic shifts associated with degradation are larger for the daughter compounds than for the parent compounds, suggesting that daughter compound degradation is more easily demonstrated by use of isotope ratios than parent compound degradation. However, the possible simultaneous formation and degradation of these intermediate metabolites complicates the interpretation of isotope data, since both processes alters the isotope ratio.

8.6.3 Phenoxy acids

The experiences with isotope ratios for phenoxy acids is limited, and it is not known if phenoxy acids are subject to isotopic fractionation during degradation. Different practical aspects are also against the use of isotope ratios for phenoxy acids.

Phenoxy acids are polar compounds, which are normally derivatized prior to GC analysis in order to improve peak shapes and resolution. However, the derivatization reaction could be associated with isotope fractionation and adds one or more extra carbon atoms to the molecule, which alters the isotope ratio.

Concentrations of phenoxy acids at contaminated sites are often relatively low (μg/L level) compared to specific compound concentrations found at hydrocarbon or chlorinated solvent sites. The detection limit of isotope analyses may therefore limit the application of isotope ratios at many phenoxy acid contaminated sites.

The online coupling of LC and IRMS and improved method detection limits may, however, make the application of isotope ratios feasible for phenoxy acids as well as other polar compounds in the future.

8.7 Indicators of specific compound degradation: Enantiomeric fractions

8.7.1 Hydrocarbons and chlorinated solvents

The majority of hydrocarbons and chlorinated hydrocarbons are not chiral, which is why the application of enantiomeric fractions is not an option.

8.7.2 Phenoxy acids

The phenoxypropionic acids, MCPP and dichlorprop, are chiral compounds, while the other phenoxy acid herbicides such as MCPA and 2,4-D are achiral. At a number of phenoxypropionic acid contaminated sites, the source can be assumed to have enantiomeric fractions of 0.5 for all phenoxypropionic acids. In these cases, a deviation from this value in the plume will indicate degradation independent of the flowpath, although a trend along a flowline may even more convincingly evidence the occurrence of biodegradation. When the source term cannot be predicted, it is necessary to have a change along a flowline in order to demonstrate *in situ* degradation. It should be noted, however, that phenoxy acid degradation is not always enantioselective.

Quantification of degradation is also possible based on changes in enantiomeric fractions, but require supportive microcosm studies to be performed. On the other hand, the demonstration of degradation potential at the site by way of microcosm degradation studies could serve as an additional line of evidence in the overall argumentation.

Measurement of phenoxy acid enantiomers is not a standard analysis, but is basically not different from other analyses of organic compounds. The only difference is the use of a chiral column, which is not a large problem, since chiral columns are commercially available for GC, LC, and CE.

9 Conclusion

In this thesis, a number of *in situ* indicators were evaluated in relation to their use in the demonstration of biodegradation. The *in situ* indicators are very different in terms of simplicity of application and interpretation, universal validity, analytical limitations, and costs. Therefore, the suitability of the different *in situ* indicators for the demonstration of field scale biodegradation is compound and site specific.

The evaluation focused on three basically different groups of compounds: Petroleum hydrocarbons, chlorinated solvents, and phenoxy acid herbicides.

9.1 Hydrocarbons

At a typical hydrocarbon field site, the hydrocarbons will serve as the primary substrate for the indigenous microorganisms, although some specific compounds may escape degradation. Some indicators can only provide information about bulk hydrocarbon degradation, while other indicators provide evidence for the degradation of single specific compounds.

For the demonstration of bulk hydrocarbon degradation, the traditional *in situ* indicator is the consumption of electron acceptors and production of metabolic by-products. Additional indication of bulk hydrocarbon degradation is the production of DIC and alkalinity, and the change in the $^{13}C/^{12}C$ isotope ratio of DIC. All of these parameters can be balanced with the observed consumption of electron acceptors. However, the use of DIC and alkalinity is more straightforward than the use of DIC isotope ratios.

Initial indication of specific compound degradation may be obtained from the possible change in compound ratios along a flowline for compounds of similar physical and chemical properties. For a range of hydrocarbons the measurement of specific metabolites can provide unequivocal evidence for the degradation of the corresponding parent hydrocarbons, although at a qualitative level. However, there are some important exceptions such as benzene for which no specific metabolites can be identified. The use of isotope ratios on the other hand, can potentially prove the degradation of any specific hydrocarbon, while the limitations associated isotope ratios are primarily related to the relatively high method detection limits in combination with the requirement of a relatively large concentration change along a flowline.

9.2 Chlorinated solvents

Chlorinated solvents should be regarded as specific compounds. The only bulk parameter that may change due to their degradation is the chloride concentration, while bulk parameters such as concentrations of electron acceptors, DIC or alkalinity will not be directly affected.

Traditionally, the distribution and concentrations of the series of daughter products down to complete dechlorination has been used to verify the degradation of chlorinated solvents. With the inclusion of the completely dechlorinated metabolites (e.g. ethene) and noting the possible existence of some daughter products as impurities in the parent compounds, this approach will probably provide the most unequivocal indication of the degradation of chlorinated solvents. Isotope ratios may provide an additional indication of degradation, but are not easily interpreted for the intermediate daughter compounds.

9.3 Phenoxy acids

Phenoxy acid herbicides are specific compounds, which will typically constitute a minor part of the bulk contaminant plume in terms of concentrations, but may be the critical compounds in terms of environmental impact. Their possible degradation will not significantly affect the concentrations of electron acceptors, DIC or alkalinity, since these parameters are controlled by the degradation of bulk contamination.

The initial metabolites of phenoxy acid degradation resulting from either sidechain cleavage or dechlorination are too unspecific for the use as indicator metabolites, because they can be present in significant amounts in the parent herbicides, which in the early years of production might contain more the 30% impurities.

On the other hand, the impurity/parent herbicide ratios are useful *in situ* indicators. Demonstration of biodegradation can by this approach be obtained from a comparison of impurity/parent herbicide ratios with the worst-case ratios (the largest possible ratios in the absence of biodegradation) or from the observation of a change in the ratio along a flowline.

The change in enantiomeric fraction along a flowline or in comparison with the source, which in many cases can be assumed to have a racemic composition, is another useful and easily applied *in situ* indicator.

9.4 Final remarks

All of the evaluated approaches are potential *in situ* indicators of biodegradation, but a number of factors influence the applicability and suitability of the different

indicators at different field sites. Some of the identified limitations are related to inherent compound properties, while others are related to the analysis, and analytical improvements may therefore increase the applicability of some of the *in situ* indicators in the future.

Overall, a more confident and exhaustive demonstration of field scale biodegradation is obtained from the combined evidence provided by different *in situ* indicators.

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